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(54) Title: IMPROVED HUMANIZED IMMUNOGLOBULINS

(57) Abstract

Novel humanized immunoglobulins having one or more complementarity determining regions (CDR's) and possible additional amino acids from a donor immunoglobulin and a framework region from an accepting human immunoglobulin are provided for a number of antigens.

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+ DESIGNATIONS OF "SU"

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IMPROVED HUMANIZED IMMUNOGLOBULINS

Field of the Invention

The present invention relates generally to the combination of recombinant DNA and monoclonal antibody technologies for developing novel therapeutic agents and, more particularly, to the production of non-immunogenic antibodies having strong affinity for a predetermined antigen.

Background of the Invention

The advent of monoclonal antibody technology in the mid 1970's heralded a new age of medicine. For the first time, researchers and clinicians had access to essentially unlimited quantities of uniform antibodies capable of binding to a predetermined antigenic site and having various immunological effector functions. These proteins, known as "monoclonal antibodies" were thought to hold great promise in, e.g., the removal of harmful cells in vivo. Indeed, the clinical value of monoclonal antibodies seemed limitless for this use alone.

Unfortunately, the development of appropriate therapeutic products based on these proteins has been severely hampered by a number of drawbacks inherent in monoclonal antibody production. For example, most monoclonal antibodies are mouse derived, and thus do not fix human complement well. They also lack other important immunoglobulin functional characteristics when used in humans.

Perhaps most importantly, non-human monoclonal antibodies contain substantial stretches of amino acid sequences that will be immunogenic when injected into a human patient. Numerous studies have shown that after injection of a foreign antibody, the immune response mounted by a patient can be quite strong, essentially eliminating the antibody's therapeutic utility after an initial treatment. Moreover, as increasing numbers of different mouse or other antigenic (to

humans) monoclonal antibodies can be expected to be developed to treat various diseases, after one or several treatments with any non-human antibodies, subsequent treatments, even for unrelated therapies, can be ineffective or even dangerous in themselves, because of cross-reactivity.

While the production of so called "chimeric antibodies" (e.g., mouse variable regions joined to human constant regions) has proven somewhat successful, a significant immunogenicity problem remains. Moreover, efforts to immortalize human B-cells or generate human hybridomas capable of producing human immunoglobulins against a desired antigen have been generally unsuccessful, particularly with many important human antigens. Most recently, recombinant DNA technology has been utilized to produce immunoglobulins which have human framework regions combined with complementarity determining regions (CDR's) from a donor mouse or rat immunoglobulin (see, e.g., EPO Publication No. 0239400). These new proteins are called "reshaped" or "humanized" immunoglobulins and the process by which the donor immunoglobulin is converted into a human-like immunoglobulin by combining its CDR's with a human framework is called "humanization". Humanized antibodies are important because they bind to the same antigen as the original antibodies, but are less immunogenic when injected into humans.

However, a major problem with humanization procedures has been a loss of affinity for the antigen (Jones et al., Nature, 321, 522-525 (1986)), in some instances as much as 10-fold or more, especially when the antigen is a protein (Verhoeyen et al., Science, 239, 1534-1536 (1988)). Loss of any affinity is, of course, highly undesirable. At the least, it means that more of the humanized antibody will have to be injected into the patient, at higher cost and greater risk of adverse effects. Even more critically, an antibody with reduced affinity may have poorer biological functions, such as complement lysis, antibody-dependent cellular cytotoxicity, or virus neutralization. For example, the loss of affinity in the partially humanized antibody

HuVHCAMP may have caused it to lose all ability to m diate compl ment lysis (see, Riechmann et al., Nature, 332, 323-327 (1988); Table 1).

Thus, there is a need for humanized antibodies specifically reactive with strong affinity to predetermined antigens. These humanized immunoglobulins should remain substantially non-immunogenic in humans, yet be easily and economically produced in a manner suitable for therapeutic formulation and other uses. The present invention fulfills these and other needs.

Summary of the Invention

The present invention provides novel compositions useful, for example, in the treatment of T-cell mediated human disorders, the compositions containing human-like immunoglobulins specifically capable of inhibiting the binding of human IL-2 to its receptor and/or capable of binding to the p75 protein of human IL-2 receptors. The immunoglobulins can have two pairs of light chain/heavy chain complexes, typically at least one chain comprising mouse complementarity determining regions functionally joined to human framework region segments. For example, mouse complementarity determining regions, with or without additional naturally-associated mouse amino acid residues, can be used to produce human-like antibodies capable of binding to the p75 protein at affinity levels stronger than about 10^7 M^{-1}. These humanized immunoglobulins will also be capable of blocking the binding of the CDR-donating mouse monoclonal antibody to p75.

The present invention also provides novel compositions useful, for example, in the treatment of HSV mediated human disorders, the compositions containing humanized immunoglobulins specifically capable of blocking the binding of HSV to its receptors and/or capable of binding to the HSV specific proteins. The immunoglobulins can have two pairs of light chain/heavy chain complexes, at least one chain comprising one or more mouse complementarity determining regions functionally joined to human framework region

segm nts. For example, mouse complementarity determining regions, with or without additional naturally-associated mouse amino acid residues, can be introduced into human framework regions to produce humanized immunoglobulins capable of binding to the HSV surface proteins at affinity levels stronger than about 10^7 M^{-1}. These humanized immunoglobulins will also be capable of blocking the binding of the CDR donating mouse monoclonal antibody to HSV. These humanized immunoglobulins may be utilized alone in substantially pure form, or together with an antiviral agent, such as acyclovir or a cytotoxic agent active at viral surfaces. All of these compounds will be particularly useful in treating HSV mediated disorders.

The present invention further provides novel compositions useful, for example, in the treatment of myeloid leukemia-related human disorders, the compositions containing humanized immunoglobulins specifically capable of binding to CD33 antigen. The immunoglobulins can have two pairs of light chain/heavy chain complexes, at least one chain comprising one or more mouse complementarity determining regions functionally joined to human framework region segments. For example, mouse complementarity determining regions, with or without additional naturally-associated mouse amino acid residues, can be introduced into human framework regions to produce humanized immunoglobulins capable of binding to the CD33 antigen at affinity levels stronger than about 10^7 M^{-1}. These humanized immunoglobulins will also be capable of blocking the binding of the CDR-donating mouse monoclonal antibody to CD33. These humanized immunoglobulins may be utilized alone in substantially pure form, or together with a chemotherapeutic agent such as cytosine arabinoside or daunorubicin active against leukemia cells, or complexed with a radionuclide such as iodine-131. All of these compounds will be particularly useful in treating leukemia and myeloid cell-mediated disorders.

The pres nt invention also provides novel compositions useful, for example, in the treatment of CMV-

mediated human disorders, the compositions containing humanized immunoglobulins sp cifically capable of blocking the binding of CMV to its receptors and/or capable of binding to CMV antigens. For example, mouse complementarity determining regions, with or without additional naturally-associated mouse amino acid residues, can be introduced into human framework regions to produce humanized immunoglobulins capable of binding to CMV at affinity levels stronger than about 10^7 M^{-1}. These humanized immunoglobulins will also be capable of blocking the binding of the CDR-donating mouse monoclonal antibody to CMV. These humanized immunoglobulins may be utilized alone in substantially pure form, or together with a chemotherapeutic agent such a acyclovir or ganciclovir active against CMV-infected cells, or complexed with a cytotoxic agent. All of these compounds will be particularly useful in treating CMV-mediated disorders.

The present invention further provides novel compositions useful, for example, in the treatment of human autoimmune disorders, the compositions containing humanized immunoglobulins specifically capable of binding to γ-IFN. For example, mouse complementarity determining regions, with or without additional naturally-associated mouse amino acid residues, can be introduced into human framework regions to produce humanized immunoglobulins capable of binding to γ-IFN at affinity levels stronger than about 10^7 M^{-1}. These humanized immunoglobulins will also be capable of blocking the binding of the CDR-donating mouse monoclonal antibody to γ-IFN. The humanized immunoglobulins may be utilized alone in substantially pure form, or together with a chemotherapeutic agent such as a non-steroidal anti-inflammatory drug, a corticosteroid, or an immunosuppressant. All of these compounds will be particularly useful in treating autoimmune disorders.

BRIEF DESCRIPTION OF THE FIGURES

Figure 1. Amino acid sequences (1-letter code) of the light chain (A) and heavy chain (B) variable regions of

the mouse Fd79 antibody (upper lines), compared with the humaniz d antibody (lower lines), not including signal sequences. The three CDR's in each chain are underlined. Residues in the humanized antibody framework replaced with mouse amino acids or typical human amino acids are double underlined. The number of the first position on each line is given on the left.

Figure 2. Amino acid sequences (1-letter code) of the light chain (A) and heavy chain (B) variable regions of the mouse Fd138-80 antibody (upper lines), compared with the humanized antibody (lower lines), not including signal sequences. The three CDR's in each chain are underlined. Residues in the humanized antibody framework replaced with mouse amino acids or typical human amino acids are double underlined. The number of the first position on each line is given on the left.

Figure 3. Amino acid sequences (1-letter code) of the light chain (A) and heavy chain (B) variable regions of the mouse M195 antibody (upper lines), compared with the humanized antibody (lower lines), not including signal sequences. The three CDR's in each chain are underlined. Residues in the humanized antibody framework replaced with mouse amino acids or typical human amino acids are double underlined. The number of the first position on each line is given on the left.

Figure 4. Amino acid sequences (1-letter code) of the light chain (A) and heavy chain (B) variable regions of the mouse mik-β1 antibody (upper lines), compared with the humanized antibody (lower lines), not including signal sequences. The three CDR's in each chain are underlined. Residues in the humanized antibody framework replaced with mouse amino acids or typical human amino acids are double underlined. The number of the first position on each line is given on the left.

Figure 5. Amino acid sequences (1-letter code) of the light chain (A) and heavy chain (B) variable regions of the mouse CMV5 antibody (upper lines), compared with the humanized antibody (lower lines), not including signal sequences. The three CDR's in each chain are underlined. Residues in the humanized antibody framework replaced with mouse amino acids or typical human amino acids are double underlined. The number of the first position on each line is given on the left.

Figure 6. Scheme for anchored polymerase chain reaction (PCR) cloning of the heavy and light chain variable domain cDNAs. RNA was prepared from about 10^7 hybridoma cells using the hot phenol extraction method. Briefly, cells were resuspended and vortexed in 1 ml of RNA extraction buffer (50 mM sodium acetate pH 5.2/1% SDS), extracted with 0.5 ml of phenol pH 5.2 at 65°C for 15 min, followed by another 15 min on ice. The aqueous phase was recovered and precipitated twice with ethanol. cDNA was synthesized from 10 ug of total RNA using reverse transcriptase (BRL, Betheseda, MD) and oligo-dT_{12-18} (Pharmacia, Piscatway, New Jersey) as primers. A poly(dG) tail was attached to the 3' end of the cDNA using terminal deoxynucleotide transferase (BRL) (E.Y. Loh et al., Science 243, 217 (1989)), the variable domain genes (V) were amplified using AmpliTaq (Perkin Elmer-Cetus) with the primer mc045 (TAATCTAGAATTCCCCCCCCCCCCCCCC) that hybridized to the poly(dG) tails and primers that hybridized to the constant region genes (C). For the light chain, the primer used was mc045 (TATAGAGCTCAAGCTTGGATGGTGGGAAGATGGATACAGTTGGTGC). For the heavy chain, the primer used was mc047 (TATAGAGCTCAAGCTTCCAGTGGATAGAC(CAT)GATGGGG(GC)TGT(TC)GTTTTGGC)

The sequence in parenthesis indicates a base degeneracy. The degeneracy was introduced so that the primer would be able to hybridize to most gamma chains. The amplified fragments were

th n digested with EcoRI and HindIII and cloned into pUC18 vector for sequencing.

Figure 7. Sequences of the cDNA and translated amino acid sequences of the light chain (A) and heavy chain (B) variable regions of the antibody mik-β1. The CDR sequences are underlined. The mature light chain protein begins with amino acid 23 Q and the mature heavy chain protein with amino acid 20 Q, preceded by the respective signal sequences.

Figure 8. Schematic diagram of the plasmids pVg1-dhfr (A) and pVk (B). The plasmid pVg1-dhfr contains the following parts: an approximately 4200 base pair BamHI-EcoRI fragment containing the amp and dhfr genes; a 630-bp fragment containing the human cytomegalovirus IE1 gene promoter and enhancer (Boshart et al., Cell 41, 521 (1985)) flanked at the 5' and 3' ends by EcoRI and XbaI linkers respectively; and a 2800 bp XbaI-BamHI fragment containing the human gamma-1 constant region gene with 215 bp of the preceding intron and the poly(A) signal. The plasmid pVk was similarly constructed, with a 1530-bp human kappa constant region gene replacing the gamma-1 gene and the gpt gene replacing the dhfr gene. The plasmids were constructed from the indicated parts using methods well-known in the art (see, Maniatis et al., Molecular Cloning: A Laboratory Manual, 2nd ed., Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y. (1989). For example, pVg1-dhfr was constructed from the plasmid pVg1 (commonly assigned U.S. patent application serial No. 07/590,274 filed September 28, 1990) by replacing the Hind III-Bgl II fragment containing the hyg gene with a 660 bp fragment containing the dhfr gene and extending to a Bgl II site (Simonsen et al., Proc. Natl. Acad. Sci. USA 80, 2495 (1983)).

Figure 9. Fluorocytometry of YTJB cells stained with (___) Isotype matched control antibody, (---) humanized mik-β1 antibody, (...) chimeric mik-β1 antibody. Cells were suspended in FACS buffer (PBS + 2% BSA + 0.1% azide) at approximately $5x10^6$/ml. 100 ul of cell suspension was transferred to a polystyrene tube and incubated with 100 ng of purified antibody on ice for 30 min. The cells were washed with FACS buffer and incubated with goat anti-human Ig antibody on ice for another 30 min. Then the cells were washed and incubated with FITC labeled rabbit anti-goat Ig antibody for 30 min. The cells were washed again and finally resuspended in PBS + 1% paraformaldehyde. Cells were analyzed on a FACSmate (Becton Dickinson).

Figure 10. Amino acid sequences of the light chain (A) and the heavy chain (B) of the humanized mik-β1 antibody, (lower lines) and human Lay antibody (upper lines), not including signal sequences. The three CDRs in each chain are underlined. Amino acids in the framework that have been replaced with mouse amino acids or consensus human amino acids in the humanized antibody are double underlined.

Figure 11. Oligonucleotides used in the construction of the humanized mik-β1 heavy chain (B) and light chain (A). The following pairs of oligonucleotides were mixed, extended with sequenase and cut with the indicated enzymes before ligation into the pBluescriptII ks (+) vector: wps54 and vc11 with Xba I and Sal I, vc12 and wps57 with Xba I and Sal I, vc16 and vc13 with Xba I and Kpn I, vc14 and vc15 with Xba I and Kpn I. Then the wps54-vc11 and vc12-wps57 fragments were excised with Xba I and Sal I ligated together into the Xba I site of pVg1-dhfr; and the vc16-vc13 fragments and vc14-vc15 fragments were excised with Xba I and Kpn I and ligated together into the Xba I site of pVk.

Figure 12. Competitive binding of labeled mik-β1 tracer to YTJB cells. About 10^6 YTJB cells were incubated with

3.0 ng of radio-iodinated mouse mik-β1 antibody (6 μCi/μg) and varing amounts of either unlabeled mouse mik-β1 antibody (•) or humanized mik-β1 antibody (○) in 200 ul of binding buffer (PBS + 10% fetal calf serum + 0.1% NaN_3 + 10 μg/ml mouse monoclonal Ig). After incubation for 2 hr at 0°C the cells were washed twice with binding buffer without mouse Ig and collected by centrifugation. The radioactivity bound to cells was measured and expressed as the ratio of bound/free cpm.

Figure 13. Inhibition of IL-2 stimulated proliferation of human PHA blasts by humanized mik-β1 + humanized anti-Tac antibodies. No antibody added (□), 2 ug each of humanized mik-β1 and humanized anti-Tac added (■).

Figure 14. Amino acid sequences of the heavy chain (A) and the light chain (B) of the murine and humanized Fd79 antibodies, and the heavy chain (C) and the light chain (D) of the murine and humanized Fd138-80 antibodies. The sequences of the murine antibody as deduced from the cDNA (upper lines) are shown aligned with the humanized antibody sequences (lower lines). The humanized Fd79 and Fd138-80 framework sequences are derived from Pom antibody and Eu antibody, respectively. Residues are numbered according to the Kabat system (E.A. Kabat et al., Sequences of Proteins of Immunological Interest (National Institutes of Health, Bethesda, MD) (1987). The three CDRs in each chain are boxed. Residues in the Pom or Eu framework that have been replaced with murine sequences or consensus human sequences are underlined.

Figure 15. Schematic diagram of the plasmids pVg1 (A) and pVk (B). The plasmid pVg1 was constructed from the following fragments; an approximately 4850 base pair BamHI-EcoRI fragment from the plasmid pSV2hph containing the amp and hyg genes; a 630-bp fragment containing the human cytomegalovirus IE1 gene promoter and enhancer (Boshart et al., Cell 41, 521 (1985)) flanked at the 5' and 3' ends by EcoRI and XbaI linkers respectively; and a 2800 bp XbaI-BamHI

fragment containing the human gamma-1 constant region gene with 215 bp of the preceding intron and the poly(A) signal. The plasmid pVk was similarly constructed, with a 1530-bp human kappa constant region gene replacing the gamma-1 gene and the gpt replacing the hyg gene.

Figure 16. Fluorocytometry of HSV-1 infected Vero cells stained with Fd79 (A) and Fd138-80 (B) antibodies. (. .) Isotype matched control antibody, (...) humanized antibody, (___) chimeric antibody. Vero cells were infected with HSV-1 (Δ305 mutant (F strain)) at 3 pfu/cell overnight. Cells were trypsinized at 0.5 mg/ml for 1 minute, washed extensively with PBS and resuspended in FACS buffer (PBS + 2% BSA + 0.1% azide) at approximately $5x10^6$/ml. 100 ul of cell suspension was transferred to a polystyrene tube and incubated with 100 ng of purified antibody on ice for 30 min. The cells were washed with FACS buffer and incubated with FITC labeled goat anti-human antibody (Cappel) on ice for another 30 min. The cells were washed again and finally resuspended in PBS + 1% paraformaldehyde. Cells were analyzed on a FACSmate (Becton Dickinson).

Figure 17. Neutralization of HSV-1 by Fd79 (A) and Fd138-80 (B). Serial dilutions of antibodies were mixed with 100 pfu of virus and incubated at 37°C for 1 hr. The viruses were then inoculated onto 6-well plates with confluent Vero cells and adsorbed at 37°C for 1 hr. Cells were overlayed with 1% agarose in medium and incubated for 4 days. Plaques were stained with neutral red.

Figure 18. Immunostaining of infected Vero cell monolayers to examine protection of cells from viral spread in tissue culture by (A) murine or humanized Fd79, (B) murine or humanized Fd138-80. 24-well plates of confluent Vero cells were inoculated with virus at 0.1 pfu/cell and allowed to adsorb for 2 hrs. at 37°C before adding 200 ul of 10 ug/ml antibodies in medium. At the end of 4 days, culture medium

was removed and plates were dried by placing overnight in a 37°C incubat r. To detect viral antigens, each well was incubated with 200 ul of anti-gB antibody at 0.5 ug/ml for 1 hr. at 37°C, washed twice and incubated with 200 ul of peroxidase conjugated goat anti-mouse IgG (Cappel, 1:300 dilution) for 1 hr. at 37°C. The plates were washed and then developed with the substrate 3-amino-9-ethyl-carbazole (AEC) (Sigma, St. Louis, MO) for 15 minutes at room temperature. Reaction was stopped by rinsing with water and air dried.

Figure 19. Sequences of the cDNA and translated amino acid sequences of the light chain (A) and heavy chain (B) variable regions of the antibody M195. The CDR sequences are underlined. The mature light chain protein begins with amino acid 21 D and the mature heavy chain protein with amino acid 20 E, preceded by the respective signal sequences.

Figure 20. Schematic diagram of the plasmids pVg1-dhfr (A) and pVk (B). The plasmid pVg1-dhfr contains the following parts: an approximately 4200 base pair BamHI-EcoRI fragment containing the amp and dhfr genes; a 630-bp fragment containing the human cytomegalovirus IE1 gene promoter and enhancer (Boshart et al., Cell 41, 521 (1985)) flanked at the 5' and 3' ends by EcoRI and XbaI linkers respectively; and a 2800 bp XbaI-BamHI fragment containing the human gamma-1 constant region gene with 215 bp of the preceding intron and the poly(A) signal. The plasmid pVk was similarly constructed, with a 1530-bp human kappa constant region gene replacing the gamma-1 gene and the gpt gene replacing the dhfr gene. The plasmids were constructed from the indicated parts using methods well-known in the art (see, Maniatis et al., Molecular Cloning: A Laboratory Manual, 2nd ed., Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y. (1989) and PCT/89/01578 filed April 13, 1989. For example, pVg1-dhfr was constructed from the plasmid pVg1 by replacing the Hind III-

Bgl II fragment containing the hyg gene with a 660 bp fragment containing the dhfr gene and extending to a Bgl II site (Simonsen et al., Proc. Natl. Acad. Sci. USA 80, 2495 (1983)).

Figure 21. Fluorocytometry of U937 cells stained with (. .) no antibody, (...) humanized M195 antibody, (---) chimeric M195 antibody. Cells were suspended in FACS buffer (PBS + 2% FCS + 0.1% azide) at approximately $5x10^6$/ml. 100 ul of cell suspension was transferred to a polystyrene tube and incubated with 50 ng of purified antibody on ice for 30 min. The cells were washed with FACS buffer and incubated with FITC labeled goat anti-human Ig antibody on ice for another 30 min. The cells were washed again and finally resuspended in PBS + 1% paraformaldehyde. Cells were analyzed on a FACSmate (Becton Dickinson).

Figure 22. Amino acid sequences of the light chain (A) and the heavy chain (B) of the humanized M195 antibody (lower lines) and human Eu antibody (upper lines), not including signal sequences. The three CDR's in each chain are underlined. Residues in the framework that have been replaced with mouse amino acids in the humanized antibody are double underlined.

Figure 23. Oligonucleotides used in the construction of the humanized M195 heavy chain (A; ma1-4) and light chain (B; ma5-8). The following pairs of oligonucleotides were mixed, extended with Klenow polymerase and cut with the indicated enzymes before ligation into pUC18: ma1 and ma2 with Xba I and Kpn I, ma3 and ma4 with Xba I and Kpn I, ma5 and ma6 with Xba I and Hind III, ma7 and ma8 with Xba I and Hind III. Then the ma1-ma2 and ma3-ma4 fragments were excised from pUC18 with Xba I and kpn I and ligated together into the Xba I site of pVg1-dhfr; and the ma5-ma6 and ma7-ma8 fragments were excised with Xba I and Hind III and ligated together into the Xba I site of pVk.

Figure 24. Competitive binding of labeled M195 tracer to U937 cells. About 4 x 10^5 U937 cells were incubated with 4.5 ng of radio-iodinated mouse M195 antibody (6 μci/μg) and varying amounts of either unlabeled mouse M195 antibody (•) or humanized M195 antibody (○) in 200 ul of binding buffer (PBS + 2% fetal calf serum + 0.1% sodium azide). After incubation for 2 hr at 0°C, the cells were washed twice with binding buffer and collected by centrifugation. The radioactivity bound to cells was measured and is expressed as the ratio of bound/free cpm.

Figure 25. Sequences of the cDNA and translated amino acid sequences of the light chain (A) and heavy chain (B) variable regions of the antibody CMV5. The CDR sequences are underlined. The start of the mature protein sequences are indicated by arrows, preceded by the respective signal sequences.

Figure 26. Schematic diagram of the plasmids pVg1-dhfr (A) and pVk (B). The plasmid pVg1-dhfr contains the following parts: an approximately 4200 base pair BamHI-EcoRI fragment containing the amp and dhfr genes; a 630-bp fragment containing the human cytomegalovirus IE1 gene promoter and enhancer (Boshart et al., Cell 41, 521 (1985)) flanked at the 5' and 3' ends by EcoRI and XbaI linkers respectively; and a 2800 bp XbaI-BamHI fragment containing the human gamma-1 constant region gene with 215 bp of the preceding intron and the poly(A) signal. The plasmid pVk was similarly constructed, with a 1530-bp human kappa constant region gene replacing the gamma-1 gene and the gpt gene replacing the dhfr gene. The plasmids were constructed from the indicated parts using methods well-known in the art (see, Maniatis et al., Molecular Cloning: A Laboratory Manual, 2nd ed., Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y. (1989) and commonly assigned U.S. patent application serial No. 07/181,862 filed April 15, 1988). For example, pVHP LaserJet

Series IIHPLASEII.PRSment containing the hyg gen with a 660 bp fragment containing the dhfr gene and extending to a Bgl II site (Simonsen et al., Proc. Natl. Acad. Sci. USA 80, 2495 (1983)).

Figure 27. Amino acid sequences of the light chain (A) and the heavy chain (B) of the humanized CMV5 antibody (lower lines) and human Wol antibody (upper lines), not including signal sequences. The three CDR's in each chain are underlined. Residues in the framework replaced with mouse amino acids or typical human amino acids in the humanized antibody are double underlined.

Figure 28. Oligonucleotides used in the construction of the humanized CMV5 light chain (A; jb16-jb19) and heavy chain (B; jb20-jb22). The following pairs of oligonucleotides were mixed, extended with Klenow polymerase and cut with the indicated enzymes before ligation into pUC18: jb16 and jb17 with Xba I and EcoR I, jb18 and jb19 with Xba I and EcoR I, jb20 and jb21 with Xba I and Kpn I, jb22 and jb23 with Xba I and Kpn I. Then the jb16-jb17 and jb18-jb19 fragments were excised with Xba I and Mlu I and ligated together into the Xba I site of pVk; and the jb20-jb21 and jb22-jb23 fragments were excised with Xba I and Kpn I and ligated together into the Xba I site of pVgl-dhfr.

Figure 29. Competitive binding of labeled CMV5 tracer to CMV-infected cells. Increasing amounts of mouse (•) or humanized (o) CMV5 antibody was added to CMV-infected HEL cells with tracer radio-iodinated mouse CMV5, and the amount of tracer bound to the cells was determined.

Figure 30. Sequences of the cDNA and translated amino acid sequences of the light chain (A) and heavy chain (B) variable regions of the antibody AF2. The CDR sequences are underlined. The mature light chain protein begins with

amino acid 30 N and the mature heavy chain protein with amino acid 36 Q, preceded by the respective signal sequences.

Figure 31. Schematic diagram of the plasmids pVg1-dhfr (A) and pVk (B). The plasmid pVg1-dhfr contains the following parts: an approximately 4200 base pair BamHI-EcoRI fragment containing the amp and dhfr genes; a 630-bp fragment containing the human cytomegalovirus IE1 gene promoter and enhancer (Boshart et al., Cell 41, 521 (1985)) flanked at the 5' and 3' ends by EcoRI and XbaI linkers respectively; and a 2800 bp XbaI-BamHI fragment containing the human gamma-1 constant region gene with 215 bp of the preceding intron and the poly(A) signal. The plasmid pVk was similarly constructed, with a 1530-bp human kappa constant region gene replacing the gamma-1 gene and the gpt gene replacing the dhfr gene. The plasmids were constructed from the indicated parts using methods well-known in the art (see, Maniatis et al., Molecular Cloning: A Laboratory Manual, 2nd ed., Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y. (1989) and commonly assigned U.S. patent application serial No. 07/181,862 filed April 15, 1988). For example, pVg1-dhfr was constructed from the plasmid pVg1 (commonly assigned U.S. patent application serial No. 07/590,274 filed September 28, 1990) by replacing the Hind III-Bgl II fragment containing the hyg gene with a 660 bp fragment containing the dhfr gene and extending to a Bgl II site (Simonsen et al., Proc. Natl. Acad. Sci. USA 80, 2495 (1983)).

Figure 32. Amino acid sequences of the light chain (A) and the heavy chain (B) of the humanized AF2 antibody (lower lines) and human Eu antibody (upper lines), not including signal sequences. The three CDR's in each chain are underlined. Residues in the framework that have been replaced with mouse amino acids or typical human amino acids in the humanized antibody are double underlined.

Figure 33. Oligonucleotides used in the construction of the humanized AF2 light chain (A; rh10-rh13) and heavy chain (B; rh20-23). The following pairs of oligonucleotides were mixed, extended with Klenow polymerase and cut with the indicated enzymes before ligation into pUC18: rh10 and rh11 with Xba I and Hind III, rh12 and rh13 with Xba I and Hind III, rh20 and rh21 with Xba I and EcoR I, rh22 and rh23 with Xba I and EcoR I. Then the rh10-rh11 and rh12-rh13 fragments were excised with Xba I and Hind III and ligated together into then Xba I site of pVk; and the rh20-rh21 and rh22-rh23 fragments were excised with Xba I and Xho I and ligated together into the Xba I site of pVg1-dhfr.

Figure 34. Fluorescence of HS294T cells incubated with γ-IFN plus varying concentrations of mouse AF2 antibody, and stained with an anti-HLA-D antibody.

DETAILED DESCRIPTION OF THE INVENTION

In accordance with the present invention, novel humanized immunoglobulins capable of specifically binding to predetermined antigens with strong affinity are provided. These immunoglobulins are substantially non-immunogenic in humans but have binding affinities of at least about 10^8 M^{-1}, preferably 10^9 M^{-1} to 10^{10} M^{-1}, or stronger. The humanized immunoglobulins will have a human framework and have one or more complementary determining regions (CDR's), plus a limited number of other amino acids, from a donor immunoglobulin specifically reactive with an antigen. The immunoglobulins can be produced economically in large quantities and find use, for example, in the treatment of various human disorders by a variety of techniques.

In order that the invention may be more completely understood, several definitions are set forth. As used herein, the term "immunoglobulin" refers to a protein consisting of one or more polypeptides substantially encoded by immunoglobulin genes. The recognized immunoglobulin genes include the kappa, lambda, alpha, gamma (IgG_1, IgG_2, IgG_3,

IgG_4), delta, epsilon and mu constant region genes, as well as the myriad immunoglobulin variable region genes. Full-length immunoglobulin "light chains" (about 25 Kd or 214 amino acids) are encoded by a variable region gene at the NH2-terminus (about 110 amino acids) and a kappa or lambda constant region gene at the COOH - terminus. Full-length immunoglobulin "heavy chains" (about 50 Kd or 446 amino acids), are similarly encoded by a variable region gene (about 116 amino acids) and one of the other aforementioned constant region genes, e.g., gamma (encoding about 330 amino acids).

One form of immunoglobulin constitutes the basic structural unit of an antibody. This form is a tetramer and consists of two identical pairs of immunoglobulin chains, each pair having one light and one heavy chain. In each pair, the light and heavy chain variable regions are together responsible for binding to an antigen, and the constant regions are responsible for the antibody effector functions. In addition to antibodies, immunoglobulins may exist in a variety of other forms including, for example, Fv, Fab, and $(Fab')_2$, as well as bifunctional hybrid antibodies (e.g., Lanzavecchia et al., Eur. J. Immunol. 17, 105 (1987)) and in single chains (e.g., Huston et al., Proc. Natl. Acad. Sci. U.S.A., 85, 5879-5883 (1988) and Bird et al., Science, 242, 423-426 (1988)). (See, generally, Hood et al., "Immunology", Benjamin, N.Y., 2nd ed. (1984), and Hunkapiller and Hood, Nature, 323, 15-16 (1986)).

An immunoglobulin light or heavy chain variable region consists of a "framework" region interrupted by three hypervariable regions, also called CDR's. The extent of the framework region and CDR's have been precisely defined (see, "Sequences of Proteins of Immunological Interest," E. Kabat et al., U.S. Department of Health and Human Services, (1983)). The sequences of the framework regions of different light or heavy chains are relatively conserved within a species. As used herein, a "human framework region" is a framework region that is substantially identical (about 85% or more, usually 90-95% or more) to the framework region of a naturally

occurring human immunoglobulin. The framework region of an antibody, that is the combined framework regions of the constituent light and heavy chains, serves to position and align the CDR's. The CDR's are primarily responsible for binding to an epitope of an antigen.

Chimeric antibodies are antibodies whose light and heavy chain genes have been constructed, typically by genetic engineering, from immunoglobulin variable and constant region genes belonging to different species. For example, the variable segments of the genes from a mouse monoclonal antibody may be joined to human constant segments, such as gamma 1 and gamma 3. A typical therapeutic chimeric antibody is thus a hybrid protein composed of the variable or antigen-binding domain from a mouse antibody and the constant or effector domain from a human antibody (e.g., A.T.C.C. Accession No. CRL 9688 secretes an anti-Tac chimeric antibody), although other mammalian species may be used.

As used herein, the term "humanized" immunoglobulin refers to an immunoglobulin comprising a human framework region and one or more CDR's from a non-human (usually a mouse or rat) immunoglobulin. The non-human immunoglobulin providing the CDR's is called the "donor" and the human immunoglobulin providing the framework is called the "acceptor". Constant regions need not be present, but if they are, they must be substantially identical to human immunoglobulin constant regions, i.e., at least about 85-90%, preferably about 95% or more identical. Hence, all parts of a humanized immunoglobulin, except possibly the CDR's, are substantially identical to corresponding parts of natural human immunoglobulin sequences. A "humanized antibody" is an antibody comprising a humanized light chain and a humanized heavy chain immunoglobulin. For example, a humanized antibody would not encompass a typical chimeric antibody as defined above, e.g., because the entire variable region of a chimeric antibody is non-human. One says that the donor antibody has been "humanized", by the process of "humanization", because

the resultant humaniz d antibody is expected to bind to the same antigen as the donor antibody that provides the CDR's.

It is understood that the humanized antibodies designed by the present method may have additional conservative amino acid substitutions which have substantially no effect on antigen binding or other immunoglobulin functions. By conservative substitutions is intended combinations such as gly, ala; val, ile, leu; asp, glu; asn, gln; ser, thr; lys, arg; and phe, tyr.

Humanized immunoglobulins, including humanized antibodies, have been constructed by means of genetic engineering. Most humanized immunoglobulins that have been previously described (Jones et al., op. cit.; Verhoeyen et al., op. cit.; Riechmann et al., op. cit.) have comprised a framework that is identical to the framework of a particular human immunoglobulin chain, the acceptor, and three CDR's from a non-human donor immunoglobulin chain. In one case (Riechmann et al., op. cit.), two additional amino acids in the framework were changed to be the same as amino acids in other human framework regions. The present invention includes criteria by which a limited number of amino acids in the framework of a humanized immunoglobulin chain are chosen to be the same as the amino acids at those positions in the donor rather than in the acceptor, in order to increase the affinity of an antibody comprising the humanized immunoglobulin chain.

The present invention is based in part on the model that two contributing causes of the loss of affinity in prior means of producing humanized antibodies (using as examples mouse antibodies as the source of CDR's) are:

(1) When the mouse CDR's are combined with the human framework, the amino acids in the framework close to the CDR's become human instead of mouse. Without intending to be bound by theory, we believe that these changed amino acids may slightly distort the CDR's, because they create different electrostatic or hydrophobic forc s than in the donor mouse antibody, and the distorted CDR's may not make as effective

contacts with the antigen as the CDR's did in the donor antibody;

(2) Also, amino acids in the original mouse antibody that are close to, but not part of, the CDR's (i.e., still part of the framework), may make contacts with the antigen that contribute to affinity. These amino acids are lost when the antibody is humanized, because all framework amino acids are made human.

3) Injected mouse antibodies have been reported to have a half-life in the human circulation much shorter than the half-life of normal antibodies (D. Shaw et al., J. Immunol., 138, 4534-4538 (1987)). Injected humanized antibodies will presumably have a half-life more similar to naturally occurring human antibodies, allowing smaller and less frequent doses to be given.

To avoid these problems, and to produce humanized antibodies that have a very strong affinity for a desired antigen, the present invention uses one or more of the following principles for designing humanized immunoglobulins. Also, the criteria may be used singly, or when necessary in combination, to achieve the desired affinity or other characteristics.

A principle is that as acceptor, a framework is used from a particular human immunoglobulin that is unusually homologous to the donor immunoglobulin to be humanized, or use a consensus framework from many human antibodies. For example, comparison of the sequence of a mouse heavy (or light) chain variable region against human heavy (or light) variable regions in a data bank (for example, the National Biomedical Research Foundation Protein Identification Resource) shows that the extent of homology to different human regions varies greatly, typically from about 40% to about 60-70%. By choosing as the acceptor immunoglobulin one of the human heavy (respectively light) chain variable regions that is most homologous to the heavy (respectively light) chain variable region of the donor immunoglobulin, fewer amino acids will be changed in going from the donor immunoglobulin to the

humanized immunoglobulin. Hence, and again without intending to be bound by theory, it is believed that there is a smaller chance of changing an amino acid near the CDR's that distorts their conformation. Moreover, the precise overall shape of a humanized antibody comprising the humanized immunoglobulin chain may more closely resemble the shape of the donor antibody, also reducing the chance of distorting the CDR's.

Typically, one of the 3-5 most homologous heavy chain variable region sequences in a representative collection of at least about 10 to 20 distinct human heavy chains will be chosen as acceptor to provide the heavy chain framework, and similarly for the light chain. Preferably, one of the 1-3 most homologous variable regions will be used. The selected acceptor immunoglobulin chain will most preferably have at least about 65% homology in the framework region to the donor immunoglobulin.

In many cases, it may be considered preferable to use light and heavy chains from the same human antibody as acceptor sequences, to be sure the humanized light and heavy chains will make favorable contacts with each other. In this case, the donor light and heavy chains will be compared only against chains from human antibodies whose complete sequence is known, e.g., the Eu, Lay, Pom, Wol, Sie, Gal, Ou and WEA antibodies (Kabat et al., op. cit.; occasionally, the last few amino acids of a human chain are not known and must be deduced by homology to other human antibodies). The human antibody will be chosen in which the light and heavy chain variable regions sequences, taken together, are overall most homologous to the donor light and heavy chain variable region sequences. Sometimes greater weight will be given to the heavy chain sequence. The chosen human antibody will then provide both light and heavy chain acceptor sequences. In practice, it is often found that the human Eu antibody will serve this role.

Regardless of how the acceptor immunoglobulin is chosen, higher affinity may be achieved by selecting a small number of amino acids in the framework of th humanized immunoglobulin chain to be the same as the amino acids at

those positions in the donor rather than in the acceptor. A second principle is that the following categories define what amino acids may be selected from the donor. Preferably, at many or all amino acid positions in one of these categories, the donor amino acid will in fact be selected.

Category 1: The amino acid position is in a CDR is defined by Kabat et al., op. cit.

Category 2: If an amino acid in the framework of the human acceptor immunoglobulin is unusual (i.e., "rare", which as used herein indicates an amino acid occurring at that position in less than about 20% but usually less than about 10% of human heavy (respectively light) chain V region sequences in a representative data bank), and if the donor amino acid at that position is typical for human sequences (i.e., "common", which as used herein indicates an amino acid occurring in more than about 25% but usually more than about 50% of sequences in a representative data bank), then the donor amino acid rather than the acceptor may be selected. This criterion helps ensure that an atypical amino acid in the human framework does not disrupt the antibody structure. Moreover, by replacing an unusual amino acid with an amino acid from the donor antibody that happens to be typical for human antibodies, the humanized antibody may be made less immunogenic.

All human light and heavy chain variable region sequences are respectively grouped into "subgroups" of sequences that are especially homologous to each other and have the same amino acids at certain critical positions (Kabat et al., op. cit.). When deciding whether an amino acid in a human acceptor sequence is "rare" or "common" among human sequences, it will often be preferable to consider only those human sequences in the same subgroup as the acceptor sequence.

Category 3: In the positions immediately adjacent to one or more of the 3 CDR's in the primary sequenc of the humanized immunoglobulin chain, the donor amino acid(s) rather than

acceptor amino acid may be sel cted. These amino acids are particularly likely to interact with the amino acids in the CDR's and, if chosen from the acceptor, to distort the donor CDR's and reduce affinity. Moreover, the adjacent amino acids may interact directly with the antigen (Amit et al., Science, 233, 747-753 (1986)) and selecting these amino acids from the donor may be desirable to keep all the antigen contacts that provide affinity in the original antibody.

Category 4: A 3-dimensional model, typically of the original donor antibody, shows that certain amino acids outside of the CDR's are close to the CDR's and have a good probability of interacting with amino acids in the CDR's by hydrogen bonding, Van der Waals forces, hydrophobic interactions, etc. At those amino acid positions, the donor immunoglobulin amino acid rather than the acceptor immunoglobulin amino acid may be selected. Amino acids according to this criterion will generally have a side chain atom within about 3 angstrom units of some atom in the CDR's and must contain an atom that could interact with the CDR atoms according to established chemical forces, such as those listed above.

In the case of atoms that may form a hydrogen bond, the 3 angstroms is measured between their nuclei, but for atoms that do not form a bond, the 3 angstroms is measured between their Van der Waals surfaces. Hence, in the latter case, the nuclei must be within about 6 angstroms (3 + sum of the Van der Waals radii) for the atoms to be considered capable of interacting. In many cases the nuclei will be from 4 or 5 to 6Å apart. In determining whether an amino acid can interact with the CDRs, it is preferred not to consider the last 8 amino acids of heavy chain CDR 2 as part of the CDRs, because from the viewpoint of structure, these 8 amino acids behave more as part of the framework.

Amino acids in the framework that are capable of interacting with amino acids in the CDR's, and which therefore

belong to Category 4, may be distinguished in another way. The solvent accessible surface area of each framework amino acid is calculated in two ways: (1) in the intact antibody, and (2) in a hypothetical molecule consisting of the antibody with its CDRs removed. A significant difference between these numbers of about 10 square angstroms or more shows that access of the framework amino acid to solvent is at least partly blocked by the CDRs, and therefore that the amino acid is making contact with the CDRs. Solvent accessible surface area of an amino acid may be calculated based on a 3-dimensional model of an antibody, using algorithms known in the art (e.g., Connolly, J. Appl. Cryst. 16, 548 (1983) and Lee and Richards, J. Mol. Biol. 55, 379 (1971)). Framework amino acids may also occasionally interact with the CDR's indirectly, by affecting the conformation of another framework amino acid that in turn contacts the CDR's.

The amino acids at several positions in the framework are known to be capable of interacting with the CDRs in many antibodies (Chothia and Lesk, J. Mol. Biol. 196, 901 (1987), Chothia et al., Nature 342, 877 (1989), and Tramontano et al., J. Mol. Biol. 215, 175 (1990)), notably at positions 2, 48, 64 and 71 of the light chain and 26-30, 71 and 94 of the heavy chain (numbering according to Kabat, op. cit.), and therefore these amino acids will generally be in Category 4. Typically, humanized immunoglobulins, of the present invention will include donor amino acids (where different) in category 4 in addition to these. The amino acids at positions 35 in the light chain and 93 and 103 in the heavy chain are also likely to interact with the CDRs. At all these numbered positions, choice of the donor amino acid rather than the acceptor amino acid (when they differ) to be in the humanized immunoglobulin is preferred. On the other hand, certain positions that may be in Category 4 such as the first 5 amino acids of the light chain may sometimes be chosen from the acceptor immunoglobulin without loss of affinity in the humanized immunoglobulin.

Chothia and Lesk (op. cit.) define the CDRs differently from Kabat et al. (op. cit.). Notably, CDR1 is

defined as including residues 26-32. Accordingly, Riechmann et al., (op. cit.) chose these amino acids from the donor immunoglobulins.

Computer programs to create models of proteins such as antibodies are generally available and well known to those skilled in the art (see, Levy et al., Biochemistry, 28, 7168-7175 (1989); Bruccoleri et al., Nature, 335, 564-568 (1988); Chothia et al., Science, 233, 755-758 (1986)). These do not form part of the invention. Indeed, because all antibodies have similar structures, the known antibody structures, which are available from the Brookhaven Protein Data Bank, can be used if necessary as rough models of other antibodies. Commercially available computer programs can be used to display these models on a computer monitor, to calculate the distance between atoms, and to estimate the likelihood of different amino acids interacting (see, Ferrin et al., J. Mol. Graphics, 6, 13-27 (1988)).

In addition to the above categories, which describe when an amino acid in the humanized immunoglobulin may be taken from the donor, certain amino acids in the humanized immunoglobulin may be taken from neither the donor nor acceptor, if then fall in:

Category 5: If the amino acid at a given position in the donor immunoglobulin is "rare" for human sequences, and the amino acid at that position in the acceptor immunoglobulin is also "rare" for human sequences, as defined above, then the amino acid at that position in the humanized immunoglobulin may be chosen to be some amino acid "typical" of human sequences. A preferred choice is the amino acid that occurs most often at that position in the known human sequences belonging to the same subgroup as the acceptor sequence.

Humanized antibodies generally have at least three potential advantages over mouse or in some cases chimeric antibodies for use in human therapy:

1) Because the effector portion is human, it may interact better with the other parts of the human immune system (e.g., destroy the target cells more efficiently by complement-dependent cytotoxicity (CDC) or antibody-dependent cellular cytotoxicity (ADCC)).

2) The human immune system should not recognize the framework or constant region of the humanized antibody as foreign, and therefore the antibody response against such an injected antibody should be less than against a totally foreign mouse antibody or a partially foreign chimeric antibody.

In one aspect, the present invention is directed to designing humanized immunoglobulins that are produced by expressing recombinant DNA segments encoding the heavy and light chain CDR's from a donor immunoglobulin capable of binding to a desired antigen, such as the human IL-2 receptor, attached to DNA segments encoding acceptor human framework regions. Exemplary DNA sequences designed in accordance with the present invention code for the polypeptide chains comprising heavy and light chain CDR's with substantially human framework regions shown in Figs. 1 through 4. Due to codon degeneracy and non-critical amino acid substitutions, other DNA sequences can be readily substituted for those sequences, as detailed below. In general, the criteria of the present invention find applicability to designing substantially any humanized immunoglobulin.

The DNA segments will typically further include an expression control DNA sequence operably linked to the humanized immunoglobulin coding sequences, including naturally-associated or heterologous promoter regions. Preferably, the expression control sequences will be eukaryotic promoter systems in vectors capable of transforming or transfecting eukaryotic host cells, but control sequences for prokaryotic hosts may also be used. Once the vector has been incorporated into the appropriate host, the host is maintained under conditions suitable for high level expression of the nucleotide sequences, and, as desired, the collection

and purification of the humanized light chains, heavy chains, light/heavy chain dimers or intact antibodies, binding fragments or other immunoglobulin forms may follow (see, S. Beychok, Cells of Immunoglobulin Synthesis, Academic Press, N.Y., (1979)).

Human constant region DNA sequences can be isolated in accordance with well known procedures from a variety of human cells, but preferably immortalized B-cells (see, Kabat op. cit. and WP87/02671). The CDR's for producing the immunoglobulins of the present invention will be similarly derived from monoclonal antibodies capable of binding to the predetermined antigen, such as the human IL-2 receptor, and produced by well known methods in any convenient mammalian source including, mice, rats, rabbits, or other vertebrates, capable of producing antibodies. Suitable source cells for the constant region and framework DNA sequences, and host cells for immunoglobulin expression and secretion, can be obtained from a number of sources, such as the American Type Culture Collection ("Catalogue of Cell Lines and Hybridomas," sixth edition (1988) Rockville, Maryland, U.S.A.).

In addition to the humanized immunoglobulins specifically described herein, other "substantially homologous" modified immunoglobulins to the native sequences can be readily designed and manufactured utilizing various recombinant DNA techniques well known to those skilled in the art. For example, the framework regions can vary specifically from the sequences in Figs. 1 through 4 at the primary structure level by several amino acid substitutions, terminal and intermediate additions and deletions, and the like. Moreover, a variety of different human framework regions may be used singly or in combination as a basis for the humanized immunoglobulins of the present invention. In general, modifications of the genes may be readily accomplished by a variety of well-known techniques, such as site-directed mutagenesis (see, Gillman and Smith, Gene, 8, 81-97 (1979) and S. Roberts et al., Nature, 328, 731-734 (1987)).

Substantially homologous immunoglobulin sequences are those which exhibit at least about 85% homology, usually at least about 90%, and preferably at least about 95% homology with a reference immunoglobulin protein.

Alternatively, polypeptide fragments comprising only a portion of the primary antibody structure may be produced, which fragments possess one or more immunoglobulin activities (e.g., complement fixation activity). These polypeptide fragments may be produced by proteolytic cleavage of intact antibodies by methods well known in the art, or by inserting stop codons at the desired locations using site-directed mutagenesis, such as after CH1 to produce Fab fragments or after the hinge region to produce $(Fab')_2$ fragments. Single chain antibodies may be produced by joining VL and VH with a DNA linker (see, Huston et al., op. cit., and Bird et al., op. cit.). Also because like many genes, the immunoglobulin-related genes contain separate functional regions, each having one or more distinct biological activities, the genes may be fused to functional regions from other genes (e.g., enzymes, see, commonly assigned U.S.S.N. 132,387, filed Dec. 15, 1987) to produce fusion proteins (e.g., immunotoxins) having novel properties. The nucleic acid sequences of the present invention capable of ultimately expressing the desired humanized antibodies can be formed from a variety of different polynucleotides (genomic or cDNA, RNA, synthetic oligonucleotides, etc.) and components (e.g., V, J, D, and C regions), as well as by a variety of different techniques. Joining appropriate synthetic and genomic sequences is presently the most common method of production, but cDNA sequences may also be utilized (see, European Patent Publication No. 0239400 and L. Reichmann et al., Nature, 332, 323-327 (1988)).

As stated previously, the DNA sequences will be expressed in hosts after the sequences have been operably linked to (i.e., positioned to ensure the functioning of) an expression control sequence. These expression vectors ar typically replicable in the host organisms either as episomes

or as an integral part of the host chromosomal DNA. Commonly, expression vectors will contain selection markers, e.g., tetracycline or neomycin, to permit detection of those cells transformed with the desired DNA sequences (see, e.g., U.S. Patent 4,704,362).

E. coli is one prokaryotic host useful particularly for cloning the DNA sequences of the present invention. Other microbial hosts suitable for use include bacilli, such as Bacillus subtilus, and other enterobacteriaceae, such as Salmonella, Serratia, and various Pseudomonas species. In these prokaryotic hosts, one can also make expression vectors, which will typically contain expression control sequences compatible with the host cell (e.g., an origin of replication). In addition, any number of a variety of well-known promoters will be present, such as the lactose promoter system, a tryptophan (trp) promoter system, a beta-lactamase promoter system, or a promoter system from phage lambda. The promoters will typically control expression, optionally with an operator sequence, and have ribosome binding site sequences and the like, for initiating and completing transcription and translation.

Other microbes, such as yeast, may also be used for expression. Saccharomyces is a preferred host, with suitable vectors having expression control sequences, such as promoters, including 3-phosphoglycerate kinase or other glycolytic enzymes, and an origin of replication, termination sequences and the like as desired.

In addition to microorganisms, mammalian tissue cell culture may also be used to express and produce the polypeptides of the present invention (see, Winnacker, "From Genes to Clones," VCH Publishers, N.Y., N.Y. (1987)). Eukaryotic cells are actually preferred, because a number of suitable host cell lines capable of secreting intact immunoglobulins have been developed in the art, and include the CHO cell lines, various COS cell lines, HeLa cells, preferably myeloma cell lines, etc, and transformed B-cells or hybridomas. Expression vectors for these cells can include

expression control sequences, such as an origin of replication, a promoter, an enhancer (Queen et al., Immunol. Rev., 89, 49-68 (1986)), and necessary processing information sites, such as ribosome binding sites, RNA splice sites, polyadenylation sites, and transcriptional terminator sequences. Preferred expression control sequences are promoters derived from immunoglobulin genes, SV40, Adenovirus, cytomegalovirus, Bovine Papilloma Virus, and the like.

The vectors containing the DNA segments of interest (e.g., the heavy and light chain encoding sequences and expression control sequences) can be transferred into the host cell by well-known methods, which vary depending on the type of cellular host. For example, calcium chloride transfection is commonly utilized for prokaryotic cells, whereas calcium phosphate treatment or electroporation may be used for other cellular hosts. (See, generally, Maniatis et al., Molecular Cloning: A Laboratory Manual, Cold Spring Harbor Press, (1982).)

Once expressed, the whole antibodies, their dimers, individual light and heavy chains, or other immunoglobulin forms of the present invention, can be purified according to standard procedures of the art, including ammonium sulfate precipitation, affinity columns, column chromatography, gel electrophoresis and the like (see, generally, R. Scopes, "Protein Purification", Springer-Verlag, N.Y. (1982)). Substantially pure immunoglobulins of at least about 90 to 95% homogeneity are preferred, and 98 to 99% or more homogeneity most preferred, for pharmaceutical uses. Once purified, partially or to homogeneity as desired, the polypeptides may then be used therapeutically (including extracorporeally) or in developing and performing assay procedures, immunofluorescent stainings, and the like. (See, generally, Immunological Methods, Vols. I and II, Lefkovits and Pernis, eds., Academic Press, New York, N.Y. (1979 and 1981)).

A preferred pharmaceutical composition of the present invention comprises the use of one or more of the subject antibodies in immunotoxins. Immunotoxins are

characterized by two components and are particularly useful for killing selected cells in vitro or in vivo. One component is a cytotoxic agent which is usually fatal to a cell when attached or absorbed. The second component, known as the "delivery vehicle," provides a means for delivering the toxic agent to a particular cell type, such as cells comprising a carcinoma. The two components are commonly chemically bonded together by any of a variety of well-known chemical procedures. For example, when the cytotoxic agent is a protein and the second component is an intact immunoglobulin, the linkage may be by way of heterobifunctional cross-linkers, e.g., SPDP, carbodiimide, glutaraldehyde, or the like. Production of various immunotoxins is well-known with the art, and can be found, for example in "Monoclonal Antibody-Toxin Conjugates: Aiming the Magic Bullet," Thorpe et al., Monoclonal Antibodies in Clinical Medicine, Academic Press, pp. 168-190 (1982). The components may also be linked genetically (see, Chaudhary et al., Nature 339, 394 (1989)).

A variety of cytotoxic agents are suitable for use in immunotoxins. Cytotoxic agents can include radionuclides, such as Iodine-131 or other isotopes of iodine, Yttrium-90, Rhenium-188, and Bismuth-212 or other alpha emitters; a number of chemotherapeutic drugs, such as vindesine, methotrexate, adriamycin, and cisplatinum; and cytotoxic proteins such as ribosomal inhibiting proteins like pokeweed antiviral protein, Pseudomonas exotoxin A, ricin, diphtheria toxin, ricin A chain, etc., or an agent active at the cell surface, such as the phospholipase enzymes (e.g., phospholipase C). (See, generally, WO90/07861, published July 26, 1990; "Chimeric Toxins," Olsnes and Phil, Pharmac. Ther., 25, 355-381 (1982); and "Monoclonal Antibodies for Cancer Detection and Therapy," eds. Baldwin and Byers, pp. 159-179, 224-266, Academic Press (1985).)

The delivery component of the immunotoxin will include the humanized immunoglobulins of the present invention. Intact immunoglobulins or their binding fragments, such as Fab, are preferably used. Typically, the antibodies

in the immunotoxins will be of the human IgM or IgG isotype, but other mammalian constant regions may be utilized as desired.

The humanized antibodies and pharmaceutical compositions thereof are particularly useful for parenteral administration, i.e., subcutaneously, intramuscularly or intravenously. The compositions for parenteral administration will commonly comprise a solution of the immunoglobulin or a cocktail thereof dissolved in an acceptable carrier, preferably an aqueous carrier. A variety of aqueous carriers can be used, e.g., water, buffered water, 0.4% saline, 0.3% glycine and the like. These solutions are sterile and generally free of particulate matter. These compositions may be sterilized by conventional, well known sterilization techniques. The compositions may contain pharmaceutically acceptable auxiliary substances as required to approximate physiological conditions such as pH adjusting and buffering agents, toxicity adjusting agents and the like, for example sodium acetate, sodium chloride, potassium chloride, calcium chloride, sodium lactate, human albumin, etc. The concentration of antibody in these formulations can vary widely, i.e., from less than about 0.5%, usually at or at least about 1% to as much as 15 or 20% by weight and will be selected primarily based on fluid volumes, viscosities, etc., in accordance with the particular mode of administration selected.

Thus, a typical pharmaceutical composition for injection could be made up to contain 1 ml sterile buffered water, and 1-10 mgs of immunoglobulin. A typical composition for intravenous infusion could be made up to contain 250 ml of sterile Ringer's solution, and 150 mg of antibody. Actual methods for preparing parenterally administrable compositions will be known or apparent to those skilled in the art and are described in more detail in, for example, Remington's Pharmaceutical Science, 15th ed., Mack Publishing Company, Easton, Pennsylvania (1980), which is incorporated herein by reference.

The antibodies of this invention can be frozen or lyophilized for storage and reconstituted in a suitable carrier prior to use. This technique has been shown to be effective with conventional immune globulins and art-known lyophilization and reconstitution techniques can be employed. It will be appreciated by those skilled in the art that lyophilization and reconstitution can lead to varying degrees of antibody activity loss (e.g., with conventional immune globulins, IgM antibodies tend to have greater activity loss than IgG antibodies) and that use levels may have to be adjusted to compensate.

The compositions containing the present humanized antibodies or a cocktail thereof can be administered for prophylactic and/or therapeutic treatments. In therapeutic application, compositions are administered to a patient in an amount sufficient to cure or at least partially arrest the disease and its complications. An amount adequate to accomplish this is defined as a "therapeutically effective dose." Amounts effective for this use will depend upon the severity of the infection and the general state of the patient's own immune system, but generally range from about 1 to about 200 mg of antibody per dose, with dosages of from 5 to 25 mg being more commonly used. It must be kept in mind that the materials of this invention may generally be employed in serious disease states, that is life-threatening or potentially life-threatening situations. In such cases, in view of the minimization of extraneous substances and the lower probability of "foreign substance" rejections which are achieved by the present humanized immunoglobulins of this invention, it is possible and may be felt desirable by the treating physician to administer substantial excesses of these antibodies.

In prophylactic applications, compositions containing the present immunoglobulins or a cocktail thereof are administered to a patient not already in a disease state to enhance the patient's resistance. Such an amount is defined to be a "prophylactically effective dose." In this

use, th precise amounts again depend upon the patient's state of health and general level of immunity, but generally range from 0.1 to 25 mg per dose.

Single or multiple administrations of the compositions can be carried out with dose levels and pattern being selected by the treating physician. In any event, the pharmaceutical formulations should provide a quantity of the antibody(ies) of this invention sufficient to effectively treat the patient.

For diagnostic purposes, the antibodies may either be labeled or unlabeled. Unlabeled antibodies can be used in combination with other labeled antibodies (second antibodies) that are reactive with the humanized antibody, such as antibodies specific for human immunoglobulin constant regions. Alternatively, the antibodies can be directly labeled. A wide variety of labels may be employed, such as radionuclides, fluors, enzymes, enzyme substrates, enzyme co- factors, enzyme inhibitors, ligands (particularly haptens), etc. Numerous types of immunoassays are available and are well known to those skilled in the art.

The following examples are offered by way of illustration, not by limitation.

The production of five specific humanized antibodies are described below. The antibodies are: Fd79 and Fd138-80 which respectively bind to the gB and gD glycoproteins of herpes simplex virus (Metcalf et al., Intervirology 29, 39 (1988)), M195 (Tanimoto et al., Leukemia 3, 339 (1989)) which binds to the CD33 antigen, mik-β1 (Tusdo et al., Proc. Natl. Acad. Sci. USA 86, 1982 (1989)) which binds to the p75 chain of the IL-2 receptor, and CMV5 which binds to the gH glycoprotein of cytomegalovirus.

Basically, cDNAs for the heavy chain and light chain variable domain genes of each antibody were cloned using anchored polymerase chain reactions (Loh et al., Science 243, 219 (1989)), using 3' primers that hybridized to the constant regions and contained HindIII sites, and 5' primers that hybridized to the dG tails and contained EcoRI sites (Scheme

shown in Fig. 6). The PCR amplified fragments were digested with EcoRI and HindIII and cloned into the pUC18 vector for sequencing. For each antibody, at least two heavy chain and two kappa clones were sequenced and found to have the same sequence. The deduced amino acid sequences of the mature light and heavy chain variable regions are shown in Figs. 1-5, upper lines.

In order to retain high binding affinity of the humanized antibodies, the principles and categories described above were utilized when designing the antibodies. Based on high sequence homology, human antibodies were selected to provide both the acceptor light and heavy chain human frameworks for the mouse antibodies, as follows: human Pom for Fd79, human Eu for Fd138-80, human Eu for M195, human Lay for mik-β1, and human Wol for CMV5.

The computer programs ABMOD and ENCAD (Levitt, J. Mol. Biol., 168, 595 (1983) and Zilber et al., Biochemistry 29, 10032 (1990)) was used to construct a model of the variable region of each mouse antibody. The model was used to determine the amino acids in each framework that were close enough to the CDR's to potentially interact with them (category 4 above). For each antibody, the positions found to fall in the categories (1) - (5) defined above are given in Table 1, numbered as in Figs. 1-5.

TABLE 1

Fd79 Antibody

Category	Light Chain	Heavy Chain
1	24-38, 54-50, 93-100	31-35, 50-66, 99-111
2	9, 45, 46, 83	82, 112
3	53	112
4	53	97
5	81	

Fd138-80 Antibody

Category	Light Chain	Heavy Chain
1	24-34, 50-56, 89-97	31-35, 50-66, 99-110
2	48, 63	93, 98, 111, 112, 113, 115
3	--	30, 67, 98, 111
4	36, 48, 87	27, 30, 37, 48, 67, 68, 98

M195 Antibody

Category	Light Chain	Heavy Chain
1	24-38, 54-60, 93-101	31-35, 50-66, 95-105
2	10, 52, 67, 110	93, 95, 98, 106, 107 108, 110
3	--	30, 67, 98, 106
4	40, 52, 74	27, 30, 48, 68, 98

mik-β1 Antibody

Category	Light Chain	Heavy Chain
1	24-33, 49-55, 88-96	31-35, 50-65, 98-108
2	13	84, 89, 90
3	--	30, 49
4	70	29, 30, 72, 73
5	41	1

CMV5 Antibody

Category	Light Chain	Heavy Chain
1	24-34, 50-56, 89-97	31-35, 50-66, 99-108
2	--	69, 80
3	49	30
4	49	24, 27, 28, 30, 97
5	--	5

In designing each humanized antibody, at each position the amino acid was selected to be the same as in the human acceptor sequence, unless the position fell in categories (1) - (4), in which case the amino acid from the mouse donor sequence was used, or in category (5), in which case an amino acid typical for human sequences at that position was used.

For the construction of genes for the humanized antibodies, nucleotide sequences were selected that encode the protein sequences of the humanized heavy and light chains, including signal peptides typically from the mouse antibody chains, generally utilizing codons found in the mouse sequence. Several degenerate codons were changed to create restriction sites or to remove undesirable ones. The nucleotide sequences also included splice donor signals typical for immunoglobulin genes and an XbaI site at each end. Each gene was constructed from four overlapping synthetic oligonucleotides. For each variable domain gene, two pairs of overlapping oligonucleotides on alternating strands were synthesized that encompassed the entire coding sequences as well as the signal peptide and the splice donor signal. The oligonucleotides were synthesized on an Applied Biosystems 380B DNA synthesizer. Each oligo was about 110-140 base long with a 15-20 base overlap. Double stranded DNA fragments were synthesized with Klenow or Taq polymerase or sequenase from each pair of oligonucleotides, digested with restriction enzymes, ligated to pUC18 vector and sequenced. Two fragments with the respectively correct half-sequences were then ligated into the XbaI sites of pVg1 (heavy chains of Fd79 and Fd138-80) or pVg1-dhfr (heavy chains of M195, mik-β1, CMV5) or pVk (all light chains) expression vectors in the appropriate orientations to produce the complete heavy and light chain genes. Reactions were carried out under conditions well-known in the art (Maniatis et al., op. cit.).

The heavy chain and light chain plasmids were transfected into Sp2/0 mouse myeloma cells by electroporation

and cells were selected for gpt expression. Clones were scre ned by assaying human antibody production in the culture supernatant by ELISA, and antibody was purified from the best-producing clones. Antibody was purified by passing tissue culture supernatant over a column of staphylococcal protein A-Sepharose CL-4B (Pharmacia). The bound antibodies were eluted with 0.2 M Glycine-HCl, pH 3.0 and neutralized with 1 M Tris pH 8.0. The buffer was exchanged into PBS by passing over a PD10 column (Pharmacia).

The binding of the humanized antibodies to cell types expressing the corresponding antigens was tested: HSV-infected cells for Fd79 and Fd138-80, U937 cells for M195, YTJB cells for mik-β1 and CMV-infected cells for CMV5. By fluorocytometry, the humanized antibodies bind approximately as well as the original mouse antibodies and the corresponding chimeric antibodies. Moreover, the humanized antibodies compete approximately as well as the corresponding mouse antibodies against the radiolabeled mouse antibodies for binding to the cells, so the humanized antibodies have approximately the same binding affinity as the mouse antibodies, typically within about 2-fold or better, see, e.g., Table 2.

TABLE 2

Binding affinities of murine and humanized antibodies.

	Mouse	Humanized
	K_a (M^{-1})	K_a (M^{-1})
Fd79 (anti-gB)	1.1×10^8	5.3×10^7
Fd138-80 (anti-gD)	5.2×10^7	4.8×10^7

From the foregoing, it will be appreciated that the humanized immunoglobulins of the present invention offer numerous advantages over other antibodies. In comparison to other monoclonal antibodies, the present humanized immunoglobulin can be more economically produced and contain

substantially less foreign amino acid sequences. This reduced likelihood of antigenicity after injection into a human patient represents a significant therapeutic improvement.

A detailed description of each humanized immunoglobulin follows.

Example I

Humanized Immunoglobulins to p75

In mammals, the immune response is mediated by two types of cells that interact specifically with foreign material, i.e., antigens. One of these cell types, B-cells, are responsible for the production of antibodies. The second cell class, T-cells, include a wide variety of cellular subsets controlling the in vivo function of both B-cells and a wide variety of other hematopoietic cells, including T-cells. (See, generally, Paul, W.E., ed., Fundamental Immunology, 2nd ed., Raven Press, New York (1989).)

One way in which T-cells exert this control is through the production of a lymphokine known as interleukin-2 (IL-2), originally named T-cell growth factor. IL-2's prime function appears to be the stimulation and maintenance of T-cells. Indeed, some immunologists believe that IL-2 may be at the center of the entire immune response (see, Farrar, J., et al., Immunol. Rev. 63, 129-166 (1982)).

To exert its biological effects, IL-2 interacts with a specific high-affinity membrane receptor (Greene, W., et al., Progress in Hematology XIV, E. Brown, Ed., Grune and Statton, New York (1986), at pgs. 283 ff and Waldmann, Ann. Rev. Biochem. 58, 875 (1989)). The human IL-2 receptor is a complex multichain glycoprotein, with one chain, known as the Tac peptide or alpha chain, being about 55kD in size (see, Leonard, W., et al., J. Biol. Chem. 260, 1872 (1985)). The second chain is known as the p75 or beta chain (Tsudo et al., Proc. Nat. Acad. Sci. USA, 83, 9694 (1986) and Sharon et al., Science 234, 859 (1986)). The p55 or Tac chain and the p75 chain each independently bind IL-2 with low or intermediate affinity, while the IL-2 receptor complex of both chains binds

IL-2 with high affinity. The p75 chain of the human IL-2 receptor will often be called herein simply the p75 protein.

Much of the elucidation of the human IL-2 receptor's structure and function is due to the development of specifically reactive monoclonal antibodies. In particular, one mouse monoclonal antibody, known as anti-Tac (Uchiyama, et al., J. Immunol. 126, 1393 (1981)) has been used to show that IL-2 receptors can be detected on T-cells, but also on cells of the monocyte-macrophage family, Kupffer cells of the liver, Langerhans' cells of the skin and, of course, activated T-cells. Importantly, resting T-cells, B-cells or circulating macrophages typically do not display the IL-2 receptor (Herrmann, et al., J. Exp. Med. 162, 1111 (1985)). Another antibody, mik-β1, binds to the p75 chain (Tsudo et al., Proc. Nat. Acad. Sci. USA 86, 1982 (1989)).

The anti-Tac monoclonal antibody has also been used to define lymphocyte functions that require IL-2 interaction, and has been shown to inhibit various T-cell functions, including the generation of cytotoxic and suppressor T lymphocytes in cell culture. Also, based on studies with anti-Tac and other antibodies, a variety of disorders are now associated with improper IL-2 receptor expression by T-cells, in particular adult T-cell leukemia.

More recently, the IL-2 receptor has been shown to be an ideal target for novel therapeutic approaches to T-cell mediated diseases. It has been proposed that IL-2 receptor specific antibodies, such as the anti-Tac monoclonal antibody or mik-β1, can be used either alone or as an immunoconjugate (e.g., with Ricin A, isotopes and the like) to effectively remove cells bearing the IL-2 receptor. These agents can, for example, theoretically eliminate IL-2 receptor-expressing leukemic cells, certain B-cells, or activated T-cells involved in a disease state, yet allow the retention of mature normal T-cells and their precursors to ensure the capability of mounting a normal T-cell immune response as needed. In general, most other T-cell specific agents can destroy essentially all peripheral T-cells, which limits the agents'

therapeutic efficacy. Overall, the use of appropriate monoclonal antibodies specific for the IL-2 receptor may have therapeutic utility in autoimmune diseases, organ transplantation and any unwanted response by activated T-cells. Indeed, clinical trials have been initiated using, e.g., anti-Tac antibodies (Kirkman et al., Transplant. Proc. 21, 1766 (1989) and Waldmann et al., Blood 72, 1805 (1988)).

Unfortunately, the use of anti-Tac, mik-β1 and other non-human monoclonal antibodies have certain drawbacks, particularly in repeated therapeutic regimens as explained below. Mouse monoclonal antibodies, for example, generally do not fix human complement well, and lack other important immunoglobulin functional characteristics when used in humans. Perhaps more importantly, anti-Tac, mik-β1 and other non-human monoclonal antibodies contain substantial stretches of amino acid sequences that will be immunogenic when injected into a human patient.

In accordance with the present invention, human-like immunoglobulins specifically reactive with the p75 chain of the human IL-2 receptor are provided. These immunoglobulins, which have binding affinities of at least 10^7 to 10^8 M^{-1}, and preferably 10^9 M^{-1} to 10^{10} M^{-1} or stronger, are capable of, e.g., blocking the binding of IL-2 to human IL-2 receptors. The human-like immunoglobulins will have a human-like framework and can have complementarity determining regions (CDR's) from an immunoglobulin, typically a mouse immunoglobulin, specifically reactive with an epitope on p75 protein. The immunoglobulins of the present invention, which can be produced economically in large quantities, find use, for example, in the treatment of T-cell mediated disorders in human patients by a variety of techniques.

In one aspect, the present invention is directed to recombinant DNA segments encoding the heavy and/or light chain CDR's from an immunoglobulin capable of binding to a desired epitope on the human IL-2 receptor, such as the mik-β1 monoclonal antibody. The DNA segments encoding these regions will typically be joined to DNA segments encoding appropriate

human-like framework r gions. Exemplary DNA sequences, which on expression cod for the polypeptide chains comprising the mik-β1 heavy and light chain CDRs, are included in Fig. 7. Due to codon degeneracy and non-critical amino-acid substitutions, other DNA sequences can be readily substituted for those sequences, as detailed above.

The antibodies will typically find use individually in treating a T-cell mediated disease state. Generally, where the cell linked to a disease has been identified as IL-2 receptor bearing, then the human-like antibodies capable of blocking the binding of IL-2 to the human IL-2 receptor are suitable (see, U.S.S.N. 085,707, entitled "Treating Human Malignancies and Disorders.") For example, typical disease states suitable for treatment include graft-versus-host disease and transplant rejection in patients undergoing an organ transplant, such as heart, lungs, kidneys, liver, etc. Other diseases include autoimmune diseases, such as Type I diabetes, multiple sclerosis, rheumatoid arthritis, systemic lupus erythematosus, and myasthenia gravis.

The human-like antibodies of the present invention may also be used in combination with other antibodies, particularly human monoclonal antibodies reactive with other markers on cells responsible for the disease. For example, suitable T-cell markers can include those grouped into the so-called "Clusters of Differentiation," as named by the First International Leukocyte Differentiation Workshop, Leukocyte Typing, Bernard, et al., Eds., Springer-Verlag, N.Y. (1984). A preferred use is the simultaneous treatment of a patient with a human-like antibody binding to p55 and a human-like antibody binding to p75 of the IL-2 receptor, i.e., humanized anti-Tac plus humanized mik-β1.

Human-like antibodies of the present invention can further find a wide variety of utilities in vitro. By way of example, the antibodies can be utilized for T-cell typing, for isolating specific IL-2 receptor bearing cells or fragments of the receptor, for vaccine preparation, or the like.

EXPERIMENTAL

Cloning of heavy chain and light chain cDNA.

cDNAs for the heavy chain and light chain variable domain genes were cloned using anchored polymerase chain reactions (E.Y. Loh et al., Science 243, 217 (1989)), using 3' primers that hybridized to the constant regions and contained HindIII sites, and 5' primers that hybridized to the dG tails and contained EcoRI sites (scheme shown in Fig. 6). The PCR amplified fragments were digested with EcoRI and HindIII and cloned into the pUC19 vector for sequencing. For mik-β1, two gamma-2a specific and two kappa specific clones were sequenced. The two gamma-2a clones and two kappa clones are respectively identical in sequence. The cDNA variable domain sequences and the deduced amino acid sequences are shown in Fig. 7.

Construction and expression of chimeric antibody.

Two plasmid vectors were prepared for construction and expression of the chimeric antibody genes. The plasmid pVg1-dhfr (Fig. 8A) contains a human cytomegalovirus IE1 promoter and enhancer (M. Boshart et al., Cell 41, 521 (1985)), the human genomic $C_\gamma 1$ segment including part of the preceding intron, and a dihydrofolate reductase (dhfr) gene (Simonsen et al., Proc. Natl. Acad. Sci. USA 80, 2495 (1983)) for selection. The plasmid pVk (Fig. 8B) is similar to pVg1-dhfr but contains the human genomic C_κ segment and the gpt gene. Derivatives of the mik-β1 heavy and light chain variable regions were prepared from the cDNAs by polymerase chain reaction. The 5' primers hybridized to the V regions starting at the ATG codons and contained XbaI sites; the 3' primers hybridized to the last 15 nucleotides of the J regions and contained splice donor signals and XbaI sites (see, C. Queen et al., Proc. Natl. Acad. Sci. USA 86, 10029 (1989)). The modified V regions were cloned into the XbaI sites of the respective plasmid vectors between the CMV promoter and the partial introns of the constant regions.

For expression of the chimeric antibody, the heavy chain and kappa chain plasmids were transfected into Sp2/0 mouse myeloma cells by electroporation and cells selected for gpt expression. Clones secreting a maximal amount of complete antibody were detected by ELISA. Purified chimeric mik-β1 antibody was shown to bind to YTJB cells, which express the p75 antigen, by flow cytometry (Fig. 9).

Computer modeling of humanized antibodies.

In order to retain high binding affinity in the humanized antibodies, the general procedures of Queen et al. were followed (C. Queen et al., Proc. Natl. Acad. Sci. USA 86, 10029 (1989)). The more homologous a human antibody is to the original murine antibody, the less likely will combining the murine CDRs with the human framework be to introduce distortions into the CDRs that could reduce affinity. Normally the heavy chain and light chain from the same human antibody are chosen to provide the framework seqeunces, so as to reduce the possibility of incompatibility in the assembling of the two chains. Based on sequence database (performed with the MicrorGenie Sequence Analysis Software (Beckman)), the antibody Lay was chosen to provide the framework sequences for humanization of mik-β1.

The computer program ENCAD (M. Levitt, J. Mol. Biol. 168, 595 (1983)) was used to construct a model of the mik-β1 variable region. The model was used to determine the amino acids in the mik-β1 framework that were close enough to the CDRs to potentially interact with them (category 4 below). To design the humanized light and heavy chain mik-β1 variable regions, at each position the amino acid was chosen to be the same as in the Lay antibody, unless that position fell in one or more of five categories:

(1) The position fell within a CDR,

(2) The Lay amino acid was unusual for human antibodies at that position, whereas the mik-β1 amino acid was typical for human antibodies at that position.

(3) The position was immediately adjacent to a CDR,

(4) The model described above suggested that the amino acid may be physically close to the antigen binding region (CDRs).

For positions in these categories, the amino acid from the (mouse) mik-β1 antibody was used. In addition, a position was in the fifth category if

(5) The Lay amino acid was highly unusual for human antibodies at that position, and the mik-β1 amino acid was different but also unusual. Then an amino acid typical for human antibodies at that position may be used.

The amino acids in each category are shown in Table 3. Some amino acids may be in more than one category. The final sequences of the humanized mik-β1 light and heavy chain variable domains are shown in Fig. 10, compared with the Lay sequences.

TABLE 3

Category	Light Chain	Heavy Chain
1	24-33, 49-55, 88-96	31-35, 50-65, 98-108
2	13	84, 89, 90
3		30, 49
4	70	29, 30, 72, 73
5	41	1

For the construction of genes for the humanized antibodies, nucleotide sequences were selected that encode the protein sequences of the humanized heavy and light chains, including the same signal peptides as in the mouse mik-β1 chains (Fig. 7), generally utilizing codons found in the mouse sequence. Several degenerate codons were changed to create restriction sites or to remove undesirable ones. The nucleotide sequences also included the same splice donor signals used in the chimeric genes and an XbaI site at each end. Each gene was constructed from four overlapping synthetic oligonucleotides. For ach variable domain gene, two pairs of overlapping oligonucleotides on alternating

strands were synthesized that encompassed the entire coding sequences as well as th signal peptide and the splice donor signal (Fig. 11). The oligonucleotides were synthesized on an Applied Biosystems 380B DNA synthesizer. Each oligo was about 110-140 base long with about a 20 base overlap. Double stranded DNA fragments were synthesized with sequenase from each pair of oligonucleotides, digested with restriction enzymes, ligated to pBluescriptII KS (+) (Stratagene) vector and sequenced. Two fragments with the respectively correct half-sequences were then ligated into the XbaI sites of the pVg1-dhfr or pVk expression vectors. In vitro mutagenesis was used to change an Ala amino acid originally encoded by oligonucleotide wps54 to the Glu (E) at position 1 of the humanized heavy chain (Fig. 10B) by changing the nucleotides CT to AG. Reactions were carried out under conditions well-known in the art (Maniatis et al., op. cit.)

The heavy chain and light chain plasmids were transfected into Sp2/0 mouse myeloma cells by electroporation and cells were selected for gpt expression. Clones were screened by assaying human antibody production in the culture supernatant by ElISA, and antibody was purified from the best-producing clones. Antibody was purified by passing tissue culture supernatant over a column of staphylococcal protein A-Sepharose CL-4B (Pharmacia). The bound antibody was eluted with 0.2 M Glycine-HCl, pH3.0 and neutralized with 1 M Tris PH8.0. The buffer was exchanged into PBS by passing over a PD10 column (Pharmacia).

Properties of humanized antibodies.

The humanized mik-β1 antibody was characterized in comparison to the murine and chimeric antibodies. The humanized antibody bound to YTJB cells, which express p75 chain at a high level, in a fluorocytometric analysis in a manner similar to the chimeric antibody (Fig. 9), showing that it recognizes the same p75 protein.

The affinity of the humanized antibody was determined by competition with the radio-iodinated mouse

mik-β1 antibody (Fig. 12). The binding affinities were calculated according to the methods of Berzofsky (J.A. Berzofsky and I.J. Berkower, in Fundamental Immunology (ed. W.E. Paul), Raven Press (New York), 595 (1984)). The binding affinity of the humanized mik-β1 antibody was within about 2-fold of the affinity of the mouse mik-β1 antibody.

The ability of humanized mik-β1 plus humanized anti-Tac antibody (see, WO90/07861 published July 26, 1990) to inhibit IL-2 stimulated proliferation of human lymphocytes was determined. Human mononuclear cells, collected from human blood by centrifugation on Ficoll-Paque (Pharmacia), were diluted to 2 x 10^6 cells/ml in RPMI medium + 10% fetal calf serum (FCS). A 1/200 volume of phytohemagglutinin P (Difco) was added and the cells were incubated for 4 days. The cells were incubated an additional 4 days in RPMI + 10% FCS + 10 u/ml IL-2. 10^5 of these PHA activated blasts were then incubated with or without 2 μg each of humanized mik-β1 and humanized anti-Tac in 150 μl of RPMI + 10% FCS in wells of a 96-well plate for 1 hr, to which various dilutions of IL-2 (Amgen) were then added in 50 μl medium. The cells were incubated 48 hr, 0.5 μCi methyl-^{3}H-thymidine (Amersham, 82 Ci/mmol) was added, and the cells were incubated 24 hr. Cells were harvested with a cell harvester and radioactivity determined. The combination of the antibodies greatly inhibited proliferation of the cells in response to IL-2 (Fig. 13), suggesting a combination of the antibodies will have strong immunosuppressive properties. Humanized mik-β1 plus humanized anti-Tac inhibited proliferation much more strongly than did either antibody alone.

Example II

Humanized Immunoglobulins to HSV Antigens

Herpes Simplex Virus types I and II (HSV-1 and HSV-2), are now estimated to be the second most frequent cause of sexually transmitted diseases in the world. Although completely accurate data are not available, infection estimates range from about 20 to 40% of the U.S. population.

A large number of diseases, from asymptomatic to life-threatening, ar associat d with HSV infection. Of particular clinical interest, encephalitis from HSV-1 infection and transmission of HSV-2 from a pregnant mother to her fetus are often fatal. Immunosuppressed patients are also subject to severe complications when infected with the virus.

More than 50 HSV polypeptides have been identified in HSV-infected cells, including at least seven major cell surface glycoproteins (see, Whitley, R., Chapt. 66, and Roizman and Sears, Chapt. 65, Virology, Eds. Fields et al., 2nd ed., Raven Press, N.Y., N.Y. (1990)). The specific biologic functions of these glycoproteins are not well defined, although gB and gD have been shown to be associated with cell fusion activity (W. Cai et al., J. Virol. 62, 2596 (1988) and Fuller and Spear, Proc. Natl. Acad. Sci. USA 84, 5454 (1987)). gB and gD express both type-specific and type-common antigenic determinants. Oakes and Lausch demonstrated that monoclonal antibodies against gB and gE suppress replication of HSV-1 in trigeminal ganglia (Oakes and Lausch, J. Virol. 51, 656 (1984)). Dix et al. showed that anti-gC and gD antibodies protect mice against acute virus-induced neurological disease (Dix et al., Infect. Immun. 34, 192 (1981)). Whitley and colleagues produced a panel of murine monoclonal antibodies against HSV-1 and showed that several of the antibodies protected mice against encephalitis and death following ocular inoculation with the virus (see, Koga et al., Virology 151, 385 (1986); Metcalf et al., Cur. Eye Res. 6, 173 (1987) and Metcalf et al., Intervirology 29, 39 1988)). Clone Fd79 (anti-gB) prevented encephalitis even when immunization was delayed until 48 hours post-infection. Fd79 and Fd138-80 (anti-gD) significantly reduced the severity of epithelial keratitis and lowered the frequency of persistent viral infection in an outbred mouse model.

In accordance with the present invention, humanized immunoglobulins specifically reactive with HSV related epitopes either directly on the virus or on inf cted cells are provided. These immunoglobulins, which have binding

affinities to HSV specific antigens of at least about 10^7 M^{-1}, and preferably 10^8 M^{-1} to 10^{10} M^{-1} or stronger, are capable of, e.g., protecting cells from HSV transmission. The humanized immunoglobulins will have a human framework and will have one or more complementarity determining regions (CDR's) from an immunoglobulin, typically a mouse immunoglobulin, specifically reactive with an HSV protein, such as gB and gD proteins. The immunoglobulins of the present invention, which can be produced economically in large quantities, find use, for example, in the treatment of HSV mediated disorders in human patients by a variety of techniques.

The HSVs are among the most intensively investigated of all viruses, and the HSV virion structure has been shown to contain about 33 proteins. Humanized immunoglobulins utilizing CDR's from monoclonal antibodies reactive with these proteins, particularly the eight surface glycoproteins (e.g., gB, gC, gD, gE, gG, gH and gI), represent preferred embodiments of the present invention (see, Spear, P.G., The Herpesviruses, vol. 3, pp. 315-356 (1984) (Roizman, B., ed), Plenum Press, N.Y., N.Y. and Spear, P.G., Immunochemistry of Viruses. The Basis for Serodiagnosis and Vaccines, pp. 425-446 (1985) (Neurath, A.R., eds.), Amsterdam: Elsevier).

In one aspect, the present invention is directed to recombinant DNA segments encoding the heavy and/or light chain CDR's from an immunoglobulin capable of binding to a desired epitope of an HSV protein, such as monoclonal antibodies reactive with HSV gB and gD glycoproteins. The DNA segments encoding these regions will typically be joined to DNA segments encoding appropriate humanized framework regions. Exemplary DNA sequences code for the polypeptide chains comprising the heavy and light chain hypervariable regions (with human framework regions) from monoclonal antibodies Fd79 and Fd138-80, shown in Fig. 14. Due to codon degeneracy and non-critical amino-acid substitutions, other DNA sequences can b readily substituted for those sequences, as detailed below.

Any humanized immunoglobulins of the present invention may also be used in combination with other

antibodies, particularly humanized antibodies reactive with different HSV antigens. For example, suitable HSV antigens to which a cocktail of humanized immunoglobulins may react include gC, gE, gF, gG and gH (see, Rector, J. et al., Infect. Immun. 38, 168 (1982) and Fuller, A. et al., J. Virol. 63, 3435 (1989)).

The antibodies can also be used as separately administered compositions given in conjunction with acyclovir or other antiviral agents. Typically, the agents may include idoxuridine or trifluorothymidine, but numerous additional agents (e.g., vidarabine) well-known to those skilled in the art for HSV treatment may also be utilized (see, Corey, L., op. cit.). A preferred pharmaceutical composition of the present invention comprises the use of the subject immunoglobulins in immunotoxins to kill cells infected by HSV.

These humanized antibodies can further find a wide variety of utilities in vitro. By way of example, the antibodies can be utilized for detection of HSV antigens, for isolating specific HSV infected cells or fragments of the virus, for vaccine preparation, or the like.

EXPERIMENTAL

Cloning of heavy chain and light chain cDNA.

cDNAs for the heavy chain and light chain variable domain genes were cloned using anchored polymerase chain regions (E.Y. Loh et al., Science 243, 217 (1989)), using 3' primers that hybridized to the constant regions and contained HindIII sites, and 5' primers that hybridized to the dG tails and contained EcoRI sites (scheme shown in Fig. 6). This method yields clones with authentic variable domain sequences, in contrast to other methods using mixed primers designed to anneal to the variable domain sequence (J.W. Larrick et al., Bio/Technology 7, 934 (1989) and Y.L. Chiang et al., BioTech. 7, 360 (1989)). The PCR amplified fragments were digested with EcoRI and HindIII and cloned into the pUC18 vector for sequencing. For Fd79, two gamma-1 specific and 5 kappa specific clones were sequenced. The two gamma-1 specific

clones are identical in sequence. This heavy chain cDNA fragment encodes a signal peptide of 19 amino acids, a V region in mouse heavy chain subgroup IIIB, a D segment, and a J_H1 segment with 4 alterations compared to the genomic J_H1 sequence. The deduced amino acid sequence is shown in Fig. 14A.

The five kappa specific clones belong to two groups. Two clones are identical and encode a kappa chain in which the conserved amino acid 23 cysteine has been substituted by a tyrosine, probably representing the non-productive allele. The other three clones have an identical sequence encoding a signal peptide sequence of 20 amino acids, a V region in mouse kappa chain subgroup III, and a J_k2 segment with a single alteration compared to the genomic J_k2 sequence (Fig. 14B). The validity of the heavy chain and the kappa chain sequences was subsequently confirmed by the construction and expression of a chimeric antibody as discussed below.

The heavy chain and the kappa chain of Fd138-80 were cloned similarly. Three clones each of the heavy chain and the kappa chain were sequenced. All three heavy chain clones have an identical sequence encoding a signal peptide sequence of 19 amino acids, a V region in mouse heavy chain subgroup II, a D segment and the J_H3 segment (Fig. 14C). The three kappa clones are also identical in sequence. This light chain fragment encodes a signal peptide sequence of 20 amino acids, a V region gene in mouse kappa chain subgroup V and the J_k5 segment (Fig. 14D). Both chains shown no irregularities in coding sequence; their validity was subsequently confirmed by construction and expression of a chimeric antibody.

Construction and expression of chimeric antibodies.

Two plasmid vectors were prepared for construction and expression of the chimeric antibody genes. The plasmid pVg1 (Fig. 15A) contains a human cytomegalovirus IE1 promoter and enhancer (M. Boshart et al., Cell 41, 521 (1985)), the human genomic $C_\gamma1$ segment including part of the preceding intron, and the hygromycin gene (Blochlinger et al., Mol.

Cell. Biol. 4, 2929 (1984)) for selection. The plasmid pVk (Fig. 15B) is similar to pVg1 but contains the human genomic C_κ segment and the gpt gene. Derivatives of the Fd79 and Fd138-80 heavy and light chain variable regions were prepared from the cDNAs by polymerase chain reaction. The 5' primers hybridized to the V regions starting at the ATG codons and contained XbaI sites; the 3' primers hybridized to the last 15 nucleotides of the J regions and contained splice donor signals and XbaI sites (see, C. Queen et al., Proc. Natl. Acad. Sci. USA 86, 10029 (1989)). The modified V regions were cloned into the XbaI sites of the respective plasmid vectors between the CMV promoter and the partial introns of the constant regions.

For expression of the chimeric antibodies, the heavy chain and kappa chain plasmids were transfected into Sp2/0 mouse myeloma cells by electroporation and cells selected for gpt expression. Clones secreting a maximal amount of complete antibody were detected by ELISA. Purified chimeric Fd79 and Fd138-80 antibodies were shown to bind to HSV-1 infected vero cells by flow cytometry. Viral neutralization assays also indicated that the chimeric antibodies retain the neutralization activities of the murine antibodies (data not shown, but see below for similar results with humanized antibodies).

Computer modeling of humanized antibodies.

In order to retain high binding affinity in the humanized antibodies, the general procedures of Queen et al. were followed (C. Queen et al., Proc. Natl. Acad. Sci. USA 86, 10029 (1989)). The more homologous a human antibody is to the original murine antibody, the less likely will combining the murine CDRs with the human framework be to introduce distortions into the CDRs that could reduce affinity. Normally the heavy chain and light chain from the same human antibody are chosen to provide the framework sequences, so as to reduce th possibility of incompatibility in the assembling of the two chains. Based on sequence homology search against

the NBRF protein sequence database (performed with the MicroGenie Sequence Analysis Software (Beckman)), the antibody Pom was chosen to provide the framework sequences for humanization of Fd79.

The computer program ENCAD (Levitt, J. Mol. Biol. 168, 595 (1983)) was used to construct a model of the Fd79 variable region. Inspection of the refined model of murine Fd79 revealed two amino acid residues in the framework that are close enough to have significant contacts with the CDR residues (Table 4). Lys in light chainNBRF position 49 has contacts with 3 amino acids in CDR2 of the light chain (L50 Tyr, L53 Asn, L55 Glu) and 2 amino acids in CDR3 of the heavy chain (H99 Asp, H100 Tyr). Leu in heavy chain position 93 also shows interactions with 2 amino acids in CDR2 of the heavy chain (H35 Ser, H37 Val) and an amino acid in CDR3 of the heavy chain (H100C Phe). Hence, L49 Lys and H93 Leu were retained in the construction of humanized Fd79, as their replacement with human Pom framework residues would be likely to introduce distortions into the CDRs. Also, 7 other residues in the Pom framework (5 in the light chain and 2 in the heavy chain) were substituted with common human residues (identical to the murine Fd79 sequence in 6 of the choices) because of their rare occurrence in other human antibodies. The elimination of unusual amino acids in the framework may further reduce immunogenicity. The murine Fd79 sequences and the corresponding humanized sequences are shown in Fig. 14A, B. Substituted residues in the Pom framework are underlined.

TABLE 4

Residues in the framework sequence showing contacts with residues in the hypervariable regions.

Residue No.[1]	Amino Acid	Contacting CDR residues[2]
Fd79		
L49	Lys	L50Y, L53N, L55E, H99D, H100Y
H93	Leu	H35S, H37V, H100CF
Fd138-80		
L36	His	L34V, L89Q
H27	Tyr	H32H, H34I
H30	Tyr	H32H, H53R
H48	Phe	H63F
H66	Lys	H63F
H67	Ala	H63F

1. The amino acid residues are numbered according to the Kabat system (E.A. Kabat et al., Sequences of Proteins of Immunological Interest, National Institutes of Health, Bethesda, MD (1987)): the first letter (H or L) stands for the heavy chain or light chain. The following number is the residue number. The last letter is the amino acid one letter code.

2. The hypervariable regions are defined according to Kabat: Light chain CDR1: residue 24-34; CDR2: 50-56; CDR3: 89-97. Heavy chain CDR1: 31-35; CDR2: 50-65; CDR3: 95-102.

Similarly, the murine heavy chain and light chain sequences of Fd138-80 were subjected to sequence homology search against the NBRF protein sequence database. The sequences of the human antibody Eu were selected to provide the framework sequences for humanized Fd138-80. Inspection of a computer-generated model of Fd138-80 revealed 6 amino acid residues in the framework that are close enough to have important contacts with CDR residues. The residues and their contacting counterparts are listed in Table 4; these murine residues were retained in the construction of humanized Fd138-80. Two other residues (L87 Phe and H37 Met) show significant contacts with L98 Phe, which is immediately adjacent to CDR3,

so these two mouse residues were also retained. Eight amino acids in the Eu framework (2 in the light chain and 6 in the heavy chain) were substituted with the murine residues (which are also consistent with the human consensus residues) because of their rare occurrence in other human antibodies. The murine Fd138-80 sequences and the corresponding humanized sequences are shown in Fig. 14C. Substituted residues in the Eu framework are underlined.

For the construction of genes for the humanized antibodies, nucleotide sequences were selected that encode the protein sequences of the humanized heavy and light chains, including the signal peptides, generally utilizing codons found in the mouse sequence. Several degenerate codons were changed to create restriction sites or to remove undesirable ones. The nucleotide sequences also included the same splice donor signals used in the chimeric genes and an XbaI site at each end. Each gene was constructed from four overlapping synthetic oligonucleotides. For each variable domain gene, two pairs of overlapping oligonucleotides on alternating strands were synthesized that encompassed the entire coding sequences as well as the signal peptide and the splice donor signal. The oligonucleotides were synthesized on an Applied Biosystems 380B DNA synthesizer. Each oligo was about 110-140 bases long with a 15 base overlap. Double stranded DNA fragments were synthesized with Klenow polymerase, digested with restriction enzymes, ligated to pUC18 vector and sequenced. The two fragments with the correct sequences were then ligated into the XbaI sites of pVg1 or pVk expression vectors.

The synthetic genes were then cloned into the pVg1 and pVk expression vectors. For each humanized antibody constructed, the heavy chain and light chain plasmids were transfected into Sp2/0 mouse myeloma cells by electroporation and cells were selected for gpt expression. Clones were screened by assaying human antibody production in the culture supernatant by ELISA, and antibody was purified from the best-producing clones. Antibodies were purified by passing tissue

culture supernatant over a column of staphylococcal protein A-Sepharose CL-4B (Pharmacia). The bound antibodies were eluted with 0.2 M Glycine-HCl, pH3.0 and neutralized with 1 M Tris PH8.0. The buffer was exchanged into PBS by passing over a PD10 column (Pharmacia).

Properties of humanized antibodies.

The humanized Fd79 and Fd138-80 antibodies were characterized in comparison to the murine and chimeric antibodies. Both humanized antibodies bind to Vero cells infected with HSV-1 in a fluorocytometric analysis in a manner similar to the chimeric antibodies (Fig. 16), suggesting that they recognize their respective viral antigens. To more quantitatively assess the binding activity, radioiodinated murine antibodies were bound to virally infected cells and Scatchard analysis performed.

The affinities of the humanized antibodies were determined by competition with the iodinated antibodies. Vero cells infected with HSV-1 were used as source of gB and gD antigens. Increasing amounts of competitor antibody (mouse or humanized) were added to 1.5 ng of radioiodinated tracer mouse antibody (2uCi/ug) and incubated with 4 x 10^5 infected Vero cells in 0.2 ml of binding buffer (PBS + 2% FCS + 0.1% azide) for 1 hr. at 4°C. Cells were washed and pelleted, and their radioactivities were measured. The concentrations of bound and free tracer antibody were calculated. The binding affinities were calculated according to the methods of Berzofsky (J.A. Berzofsky and I.J. Berkower, in Fundamental Immunology (ed. W.E. Paul), Raven Press (New York), 595 (1984)).

The measurements indicate that there is no significant loss of binding affinities in the humanized antibodies (Table 5). Specifically, there is an approximately 2-fold decrease in affinity in humanized Fd79 compared to the murine Fd79 (Ka of 5.3 x 10^7 M^{-1} vs. 1.1 x 10^8 M^{-1}). The affinity of humanized Fd138-80 is comparable to that of the murine antibody (Ka of 4.8 x 10^7 M^{-1} vs 5.2 x 10^7 M^{-1}).

TABLE 5

Binding affinities of murine and humanized antibodies.

	Mouse	Humanized
	K_a (M^{-1})	K_a (M^{-1})
Fd79 (anti-gB)	1.1×10^8	5.3×10^7
Fd138-80 (anti-gD)	5.2×10^7	4.8×10^7

Murine Fd79 and Fd138-80 have been shown to neutralize HSV-1 in vitro without complement (J. Koga et al., Virology 151, 385 (1986)), so the neutralizing activities of the humanized antibodies were compared with the mouse antibodies. Serial dilutions of equal quantities of murine and humanized antibodies were incubated with virus for 1 hr. before inoculation onto Vero cells. After 4 days, cells were stained with neutral red to visualize plaques. Results from these plaque reduction assays indicated that both humanized Fd79 and Fd138-80 neutralize virus as efficiently as their murine counterparts (Figs. 17A and B). Both humanized and murine Fd79 cause a 90% reduction of plaques at an antibody concentration of 10 nM (1.5 ug/ml). Similarly, humanized and murine Fd138-80 were able to cause a 90% plaque reduction at equivalent levels.

The antibodies were also investigated for their ability to protect cells from viral spread in tissue culture. Vero cells were inoculated with virus at 0.1 pfu/cell and allowed to adsorb for 2 hrs. at 37°C before addition of 10 ug/ml antibody. After four days, cells were stained with an anti-gB antibody for detection of viral antigens on infected cells. Results indicated that both murine and humanized Fd79 at 10 ug/ml protected culture cells from infection (Fig. 38A). However, neither murine nor humanized Fd138-80 were able to protect cells against viral spread (Fig. 18B), despite their ability to neutralize virus before inoculation. Both gB and gD are thought to be associated with cell fusion and virus infectivity (W. Cai et al., J. Virol. 62, 2596 (1988) and A.O.

Fuller and P.G. Spear, Proc. Natl. Acad. Sci. USA 84, 5454 (1987)). However, it is possible that Fd79 blocks both the infectivity and cell fusion functions of gB, while Fd138-80 blocks only the infectivity function of gD, so virus can still spread cell-to-cell.

The binding, neutralization and protection results all indicate that the humanized Fd79 and Fd138-80 antibodies have retained the binding activities and the biological properties of the murine monoclonal antibodies. The availability of humanized antibodies with specificity for HSV gB and gD, inter alia, provides an opportunity for studies of the in vivo potency and immunogenicity of humanized antibodies in treating viral diseases. The recognition by Fd79 and Fd138-80 of type-common epitopes of gB and gD (J. Koga et al., Virology 151, 385 (1986)) expands the therapeutic potential to herpes simplex virus type 2 as well as type 1.

Protection from herpes simplex virus type 2 lethal infections in mouse model by humanized Fd79 and Fd138-80.

To determine the efficacy of humanized antibodies against herpes infections in vivo, humanized antibodies were injected into mice before and after inoculation of lethal doses of HSV-2, and the mortality rates were monitored. Groups of animals were treated intraperitoneal with 0.9, 0.3 or 0.1 mg of each of humanized Fd79 or Fd138-80 at 24 hr before or 24 hr. after viral inoculation. Groups of 10 mice were challenged intranasally with lethal doses of HSV-2. Mice were monitored for three weeks. The mortality rates were shown in the following tables.

The results show that significant protection against HSV-2 infection of mice was obtained with humanized Fd79 and humanized Fd138-80.

TABLE 6

EFFECT OF PRE-TREATMENT (-24h)
WITH HSV ANTIBODY ON THE MORTALITY
OF MICE INOCULATED INTRANASALLY WITH HSV-2

Treatment	Mortality		P-Value
	Number	Percent	
Control	13/15	87	---
Placebo	13/15	87	NS
Mu Fd 138			
0.9 mg	3/10	30	<0.001
0.3 mg	5/10	50	0.01
0.1 mg	5/10	50	0.08
Hu Fd 138			
0.9 mg	1/10	10	<0.001
0.3 mg	8/10	80	NS
0.1 mg	7/10	70	NS
Mu Fd 79			
0.9 mg	0/10	0	<0.001
0.3 mg	2/10	20	<0.01
0.1 mg	4/10	40	<0.05
Hu Fd 79			
0.9 mg	1/10	10	<0.01
0.3 mg	3/10	30	0.08
0.1 mg	5/10	50	0.08

TABLE 7

EFFECT OF POST-TREATMENT (+24h)
WITH HSV ANTIBODY ON THE MORTALITY
OF MICE INOCULATED INTRANASALLY WITH HSV-2

Treatment	Mortality Number	Mortality Percent	P-Value
Control	12/15	80	---
Placebo	15/15	100	NS
Mu Fd 138			
0.9 mg	2/10	20	<0.001
0.3 mg	4/10	40	0.001
0.1 mg	5/10	50	<0.01
Hu Fd 138			
0.9 mg	3/10	30	<0.001
0.3 mg	3/10	30	<0.001
0.1 mg	9/10	90	NS
Mu Fd 79			
0.9 mg	5/10	50	<0.01
0.3 mg	3/10	30	<0.001
0.1 mg	6/10	60	<0.05
Hu Fd 79			
0.9 mg	3/10	30	<0.001
0.3 mg	3/10	30	<0.001
0.1 mg	9/10	90	NS

The use of a combination of two or more humanized antibodies in therapy is important for reducing the development of antibody resistant strains. Combination therapy of humanized antibodies with other antiviral agents such as acyclovir provides further opportunities to combat diseases when chemotherapeutic agents alone have not been effective. As Fd79 and Fd138-80 reduce the frequency of viral persistence in a murine ocular model (J.F. Metcalf et al., Cur. Eye Res. 6, 173 (1987)), the humanized antibodies, typically together with other antiviral agents, are capable of reducing episodes of recurrent genital infection, an area where traditional anti-viral agents have not been effective (L. Corey et al., N. Engl. J. Med. 306, 1313 (1982)). Incorporation of the human constant domains can also enhance

effector functions, such as antibody-dependent cellular cytotoxicity, leading to greater therapeutic efficiency in human patients.

Example III

Humanized Immunoglobulins to CD33 Antigen

There are about 10,000-15,000 new cases of myeloid (also called non-lymphocytic or granulocytic) leukemia in the U.S. per year (Cancer Facts & Figures, American Cancer Society, 1987). There are two major forms of myeloid leukemia: acute myelogenous leukemia (AML) and chronic myelogenous leukemia (CML). Despite treatment with chemotherapy, long-term survival in patients with AML is less than 10-20% (Clarkson et al., CRC Critical Review in Oncology/Hematology 4, 221 (1986)), and survival with CML and related diseases such as chronic myelomonocytic leukemia (CMML), chronic monocytic leukemia (CMMOL) and myelodysplastic syndrome (MDS) is even lower.

The p67 protein or CD33 antigen is found on the surface of progenitors of myeloid cells and of the leukemic cells of most cases of AML, but not on lymphoid cells or non-hematopoietic cells (see, Leucocyte Typing III, ed. by A.J. McMichael, Oxford University Press, pp. 622-629 (1987). Antibodies that are known to bind to the CD33 antigen include L4B3, L1B2 and MY9 (Andrews et al., Blood 62, 124 (1983) and Griffin et al., Leukemia Research 8, 521 (1984)).

Another antibody that binds to CD33 is M195 (Tanimoto et al., Leukemia 3, 339 (1989) and Scheinberg et al., Leukemia 3, 440 (1989)). The reactivity of M195 with a wide variety of cells and tissues was tested. Among normal cells, M195 was reported to bind only to some monocytes and myeloid progenitor cells. The research also reported that it does not bind to other hematopoietic cells or to non-hematopoietic tissues. M195 bound to cells of most cases of AML and all cases of CML in myeloblastic phase.

A phase I clinical trial of M195 in AML has been conducted (Scheinberg et al., Proc. ASCO 9, 207 (1990)). M195

radiolabeled with iodine-131 was found to rapidly and specifically targ t leukemic cells in both the blood and bone marrow.

In accordance with the present invention, humanized immunoglobulins specifically reactive with CD33 related epitopes are provided. These immunoglobulins, which have binding affinities to CD33 of at least about 10^7 M^{-1}, and preferably 10^8 M^{-1} to 10^{10} M^{-1} or stronger, are capable of, e.g., destroying leukemia cells. The humanized immunoglobulins will have a human framework and will have one or more complementarity determining regions (CDR's) from an immunoglobulin, typically a mouse immunoglobulin, specifically reactive with CD33 antigen. In a preferred embodiment, one or more of the CDR's will come from the M195 antibody. Importantly, M195 does not bind to the ultimate hematopoietic stem cells, so M195 used in therapy will minimally interact with and destroy those cells, which are critical for generating all blood cells. Thus, the CD33 specific immunoglobulins of the present invention, which can be produced economically in large quantities, find use, for example, in the treatment of myeloid cell-mediated disorders in human patients by a variety of techniques.

In one aspect, the present invention is directed to recombinant DNA segments encoding the heavy and/or light chain CDR's from an immunoglobulin capable of binding to a desired epitope of CD33 antigen, such as monoclonal antibodies M195, L4B3, L1B2 or MY9. The DNA segments encoding these regions will typically be joined to DNA segments encoding appropriate human framework regions. Exemplary DNA sequences, which on expression code for the polypeptide chains comprising the heavy and light chain CDR's of monoclonal antibody M195 are included in Fig. 19. Due to codon degeneracy and non-critical amino-acid substitutions, other DNA sequences can be readily substituted for those sequences, as detailed below.

The antibodies of the present invention will typically find use individually in treating hematologic malignancies. For example, typical disease states suitable

for treatment include AML, CML, CMML, CMMOL and MDS (see, generally, Hoffbrand & Pettit, Essential Haematology, Blackwell Scientific Publications, Oxford (1980)). The antibodies may also be used for bone marrow ablation prior to bone marrow transplant.

Any humanized immunoglobulins of the present invention may also be used in combination with other antibodies, particularly humanized antibodies reactive with different myeloid antigens. For example, suitable antigens to which a cocktail of humanized immunoglobulins may react include CD13, CD14, CD15, CD16 and CD34 (see, Leukocyte Typing III, op. cit., pp. 576-732).

The antibodies can also be used as separately administered compositions given in conjunction with chemotherapeutic agents. Typically, the agents may include cytosine arabinoside and daunorubicin, but numerous additional agents (e.g., 6-thioguanine) well-known to those skilled in the art for leukemia treatment may also be utilized (see, Hoffbrund & Pettit., op. cit.). A preferred pharmaceutical composition of the present invention comprises the use of the subject immunoglobulins in immunotoxins to kill leukemia cells.

Humanized antibodies of the present invention can further find a wide variety of utilities in vitro. By way of example, the antibodies can be utilized for detection of CD33 antigens, for isolating specific myeloid cells, or the like.

It will be understood that although the experiments pertain to the M195 antibody, producing humanized antibodies with high binding affinity for the CD33 antigen is also contemplated using CDR's from L4B3, L1B2, MY9 or other monoclonal antibodies that bind to an epitope of CD33.

EXPERIMENTAL

Cloning of heavy chain and light chain cDNA.

cDNAs for the heavy chain and light chain variable domain genes were cloned using anchored polymerase chain reactions (E.Y. Loh et al., Science 243, 217 (1989)), using 3'

primers that hybridized to the constant regions and contained HindIII sites, and 5' primers that hybridized to the dG tails and contained EcoRI sites (scheme shown in Fig. 6). The PCR amplified fragments were digested with EcoRI and HindIII and cloned into the pUC18 vector for sequencing. For M195, two gamma-2a specific and two kappa specific clones were sequenced. The two gamma-2a clones and two kappa clones are respectively identical in sequence. The cDNA variable domain sequences and the deduced amino acid sequences are shown in Fig. 19.

Construction and expression of chimeric antibody.

Two plasmid vectors were prepared for construction and expression of the chimeric antibody genes. The plasmid pVg1-dhfr (Fig. 20A) contains a human cytomegalovirus IE1 promoter and enhancer (M. Boshart et al., Cell 41, 521 (1985)), the human genomic Cγ1 segment including part of the preceding intron, and a dihydrofolate reductase (dhfr) gene (Simonsen et al., Proc. Natl Acad. Sci. USA 80, 2495 (1984)) for selection. The plasmid pVk (Fig. 20B) is similar to pVg1-dhfr but contains the human genomic Cκ segment and the gpt gene. Derivatives of the M195 heavy and light chain variable regions were prepared from the cDNAs by polymerase chain reaction. The 5' primers hybridized to the V regions starting at the ATG codons and contained XbaI sites; the 3' primers hybridized to the last 15 nucleotides of the J regions and contained splice donor signals and XbaI sites (see, Queen et al., Proc. Natl. Acad. Sci. USA 86, 10029 (1989)). The modified V regions were cloned into the XbaI sites of the respective plasmid vectors between the CMV promoter and the partial introns of the constant regions.

For expression of the chimeric antibody, the heavy chain and kappa chain plasmids were transfected into Sp2/0 mouse myeloma cells by electroporation and cells selected for gpt expression. Clones secreting a maximal amount of complete antibody were detected by ELISA. Purified chimeric M195

antibody was shown to bind to U937 cells, which express the CD33 antigen, by flow cytometry (Fig. 21).

Computer modeling of humanized antibodies.

In order to retain high binding affinity in the humanized antibodies, the general procedures of Queen et al. were followed (see, Queen et al., Proc. Natl. Acad. Sci. USA 86, 10029 (1989) and WO 90/07861). The more homologous a human antibody is to the original murine antibody, the less likely will combining the murine CDR's with the human framework be to introduce distortions into the CDR's that could reduce affinity. Normally the heavy chain and light chain from the same human antibody are chosen to provide the framework sequences, so as to reduce the possibility of incompatibility in the assembling of the two chains. Based on sequence homology search against the NBRF protein sequence database (performed with the MicroGenie Sequence Analysis Software (Beckman)), the antibody Eu was chosen to provide the framework sequences for humanization of M195.

The computer program ENCAD (M. Levitt, J. Mol. Biol. 168, 595 (1983)) was used to construct a model of the M195 variable region. The model was used to determine the amino acids in the M195 framework that were close enough to the CDR's to potentially interact with them (category 4 below). To design the humanized light and heavy chain M195 variable regions, at each position the amino acid was chosen to be the same as in the Eu antibody, unless that position fell in one or more of four categories:

(1) The position fell within a CDR,

(2) The Eu amino acid was unusual for human antibodies at that position, whereas the M195 amino acid was typical for human antibodies at that position,

(3) The position was immediately adjacent to a CDR,

(4) The model described above suggested that the amino acid may be physically close to the antigen binding region (CDR's).

In category (2), "unusual" is interpreted to include amino acids that occur in less than about 20% of the human sequences in the same subgroups (as defined by Kabat et al., op. cit.) as the Eu light and heavy chains, and "typical" is interpreted to include amino acids that occur in more than about 25% but generally more than 50% of the human sequences in those subgroups. For positions in these categories, the amino acid from the mouse M195 antibody was used: The amino acids in each category are shown in Table 8. Some amino acids may be in more than one category. The final sequences of the humanized M195 light and heavy chain variable domains are shown in Fig. 22, compared with the Eu sequences.

TABLE 8

Category	Light Chain	Heavy Chain
1	24-38, 54-60, 93-101	31-35, 50-66, 99-105
2	10, 52, 67, 110	93, 95, 98, 106, 107, 108, 110
3	--	30, 67, 98, 106
4	40, 52, 74	27, 30, 48, 68, 98

For the construction of genes for the humanized antibodies, nucleotide sequences were selected that encode the protein sequences of the humanized heavy and light chains, including the same signal peptides as in the mouse M195 chains (Fig. 19), generally utilizing codons found in the mouse sequence. Several degenerate codons were changed to create restriction sites or to remove undesirable ones. The nucleotide sequences also included the same splice donor signals used in the chimeric genes and an XbaI site at each end. Each gene was constructed from four overlapping synthetic oligonucleotides. For each variable domain gene,

two pairs of overlapping oligonucleotides on alternating strands were synthesized that encompassed the ntire coding sequences as well as the signal peptide and the splice donor signal (Fig. 23). The oligonucleotides were synthesized on an Applied Biosystems 380B DNA synthesizer. Each oligo was about 110-140 bases long with about a 15 base overlap. Double stranded DNA fragments were synthesized with Klenow polymerase from each pair of oligonucleotides, digested with restriction enzymes, ligated to the pUC18 vector and sequenced. Two fragments with the respectively correct half-sequences were then ligated into the XbaI sites of the pVg1-dhfr or pVk expression vectors in the appropriate orientations to produce the complete heavy and light chain genes. Reactions were carried out under conditions well-known in the art (Maniatis et al., op. cit.)

The heavy chain and light chain plasmids were transfected into Sp2/0 mouse myeloma cells by electroporation and cells were selected for gpt expression. Clones were screened by assaying human antibody production in the culture supernatant by ELISA, and antibody was purified from the best-producing clones. Antibody was purified by passing tissue culture supernatant over a column of staphylococcal protein A-Sepharose CL-4B (Pharmacia). The bound antibody was eluted with 0.2 M Glycine-HCl, pH3.0 and neutralized with 1 M Tris PH8.0. The buffer was exchanged into PBS by passing over a PD10 column (Pharmacia).

Properties of humanized antibodies.

The humanized M195 antibody was characterized in comparison to the murine and chimeric antibodies. The humanized antibody bound to U937 cells in a fluorocytometric analysis in a manner similar to the chimeric antibody (Fig. 21), showing that it recognizes the same CD33 antigen.

The affinity of the humanized antibody was determined by competition with the radio-iodinated mouse M195 antibody (Fig. 24). The binding affinities were calculated according to the methods of Berzofsky (J.A. Berzofsky and I.J.

Berkower, in Fundamental Immunology (ed. W.E. Paul), Raven Press (New York), 595 (1984)). The mouse M195 had an affinity comparable to the published value (Tanimoto et al., op. cit.) and the humanized M195 antibody had an affinity the same as the mouse M195 to within experimental error.

Humanized M195 is useful to mediate antibody-dependent cellular cytotoxicity when human effector cells and human CD33-expressing cells are used. This is analogous to other humanized antibodies, such as reported by Junghans et al., Cancer Research 50, 1495 (1990).

Unfortunately, the use of non-human monoclonal antibodies such as M195 have certain drawbacks in human treatment, particularly in repeated therapeutic regimens.

Example IV

Humanized Immunoglobulins to CMV Antigens

Cytomegalovirus is a major pathogen of immunocompromised individuals, especially bone marrow transplant recipients, organ tansplant recipients, and AIDS patients (see, generally, Fields et al., Eds., Virology, 2nd ed., Raven Press, New York pp. 1981-2010 (1990)). Approximately 15% of bone marrow transplant patients develop CMV pneumonia, with an 85% mortality rate (Meyers, Rev. Inf. Dis. 11 (suppl. 7), S1691 (1989)). About 10% of AIDS patients develop severe CMV disease; and congenitally acquired CMV, often with significant morbidity and mortality, affects 1% of newborns (Fields, op. cit.).

The drug ganciclovir is effective against certain forms of CMV infection, notably chorioretinitis and gastroenteritis, but is not very effective against CMV pneumonia, and it has serious toxicity. Use of pooled human imunoglobulin preparations has shown some beneficial effect for prophylaxis of CMV in bone marrow transplant patients (Meyers, op. cit.), and a combination of high-dose immune globulin and ganciclovir has been reported effective against CMV pneumonia (Emanuel et al., Trans. Proc. XIX (suppl. 7), 132 (1987)). However, the marginal effectiveness, variable

potency and high cost of commercial human immune globulin remain serious problems. Henc , there is a great need for new drugs effective against CMV.

CMV is a member of the herpesvirus family of viruses, and as such, has a large double-stranded DNA core, a protein capsid, and an outer lipid envelope with viral glycoproteins on its surface. At least 8 proteins have been detected on the envelope of CMV (Britt et al., J. Virol. 62, 3309 (1988)) and others have been predicted to exist based on the DNA sequence of CMV (Chee et al., Nature 344, 774 (1990)). Murine monoclonal antibodies have been produced against two especially significant CMV glycoproteins: gB, also called p130/55 or gp55-116, and gH, also called p86 (Rasmussen et al., Virology 163, 308 (1988) and Britt et al., op. cit.) and shown to neutralize infectivity of the virus. Three other neutralizing antibodies to gH are designated CMV5, CMV109 and CMV115. Human monoclonal antibodies to CMV have also been produced (Ehrlich et al., Hybridoma 6, 151 (1987)).

In animal models, murine monclonal antibodies have been shown effective in treating infections caused by various viruses, including members of the herpesvirus family (see, e.g., Metcalf et al., Intervirol. 29, 39 (1988)). Hence, such antibodies may be useful in treatment of CMV infections. Unfortunately, the use of non-human monoclonal antibodies such as CMV5 and CMV115 have certain drawbacks in human treatment, particularly in repeated therapeutic regimens as explained below.

Thus, there is a need for improved forms of humanized immunoglobulins specific for CMV antigen that are substantially non-immunogenic in humans, yet easily and economically produced in a manner suitable for therapeutic formulation and other uses. The present invention fulfills these and other needs.

In accordance with the present invention, humanized immunoglobulins specifically reactive with CMV and CMV-infected cells are provided. These immunoglobulins, which have binding affinities to CMV specific antigens of at least

about 10^7 M^{-1}, and preferably 10^8 M^{-1} to 10^{10} M^{-1} or stronger, are capable of, e.g., blocking CMV infection of cells. Th humanized immunoglobulins will have a human framework and will have one or more complementarity determining regions (CDR's) from an immunoglobulin, typically a mouse immunoglobulin, specifically reactive with a CMV antigen. In a preferred embodiment, one or more of the CDR's will come from the CMV5, or CMV109 or CMV115 antibodies. The immunoglobulins of the present invention, which can be produced economically in large quantities, find use, for example, in the treatment of CMV-mediated disorders in human patients by a variety of techniques.

In one aspect, the present invention is directed to recombinant DNA segments encoding the heavy and/or light chain CDR's from an immunoglobulin capable of binding to a desired epitope of a CMV antigen, such as monoclonal antibodies CMV5 or CMV115. The DNA segments encoding these regions will typically be joined to DNA segments encoding appropriate human framework regions. Exemplary DNA sequences, which on expression code for the polypeptide chains comprising the heavy and light chain CDR's of monoclonal antibody CMV5 are included in Fig. 25. Due to codon degeneracy and non-critical amino-acid substitutions, other DNA sequences can be readily substituted for those sequences, as detailed below.

Polypeptide fragments comprising only a portion of the primary antibody structure may be produced, which fragments possess one or more immunoglobulin activities (e.g., complement fixation activity). These polypeptide fragments may be produced by proteolytic cleavage of intact antibodies by methods well known in the art, or by inserting stop codons at the desired locations in the vectors pVk and pVg1-dhfr (Fig. 26) using site-directed mutagenesis, such as after CH1 to produce Fab fragments or after the hinge region to produce $(Fab')_2$ fragments. Single chain antibodies may be produced by joining VL and VH with a DNA linker (see Huston et al., op. cit., and Bird et al., op. cit.).

The antibodies of the present invention will typically find use individually in treating CMV-related disorders. For example, typical disease states suitable for treatment include CMV pneumonia, neonatal CMV infection, CMV mononucleosis and CMV-related chorioretinitis and gastroenteritis. Any humanized immunoglobulins of the present invention may also be used in combination with other antibodies, particularly humanized antibodies reactive with different CMV antigens. For example, suitable antigens to which a cocktail of humanized immunoglobulins may react include the gB and gH proteins. The antibodies can also be used as separately administered compositions given in conjunction with chemotherapeutic agents. Typically, the agents may include acyclovir or ganciclovir, but numerous additional agents well-known to those skilled in the art for CMV treatment may also be utilized. A preferred pharmaceutical composition of the present invention comprises the use of the subject immunoglobulins in immunotoxins to kill CMV-infected cells.

CMV specific humanized antibodies of the present invention can further find a wide variety of utilities in vitro. By way of example, the antibodies can be utilized for detection of CMV antigens, for isolating specific CMV-infected cells, or the like.

In particular, the same method may be used to produce a humanized CMV109, CMV115 or other anti-CMV antibody as used to produce humanized CMV5 herein.

EXPERIMENTAL

Cloning of heavy chain and light chain cDNA.

cDNAs for the heavy chain and light chain variable domain genes were cloned using anchored polymerase chain reactions (E.Y. Loh et al., Science 243, 217 (1989)), using 3' primers that hybridized to the constant regions and contained HindIII sites, and 5' primers that hybridized to the dG tails and contained EcoR I sites (scheme shown in Fig. 6). The PCR amplified fragments were digested with EcoR I and HindIII and

cloned into the pUC18 vector for sequencing. For CMV5, two gamma-2a specific and two kappa specific clones were sequenced. The two gamma-2a clones and two kappa clones are respectively identical in sequence. The cDNA variable domain sequences and the deduced amino acid sequences are shown in Fig. 25A and 25B. Similarly, by using techniques, which are well-known in the art, cDNAs for the CMV109 and CMV115 antibodies may be obtained and their sequence determined.

Construction and expression of chimeric antibody.

Two plasmid vectors were prepared for construction and expression of the chimeric antibody genes. The plasmid pVg1-dhfr (Fig. 26A) contains a human cytomegalovirus IE1 promoter and enhancer (M. Boshart et al., Cell 41, 521 (1985)), the human genomic $C\gamma 1$ segment including part of the preceding intron, and a dihydrofolate reductase (dhfr) gene (Simonsen et al., Proc. Natl. Acad. Sci. USA 80, 2495 (1983)) for selection. The plasmid pVk (Fig. 26B) is similar to pVg1-dhfr but contains the human genomic $C\kappa$ segment and the gpt gene. Derivatives of the CMV5 heavy and light chain variable regions were prepared from the cDNAs by polymerase chain reaction. The 5' primers hybridized to the V regions starting at the ATG codons and contained XbaI sites; the 3' primers hybridized to the last 15 nucleotides of the J regions and contained splice donor signals and XbaI sites (see, Queen et al., Proc. Natl. Acad. Sci. USA 86, 10029 (1989)). The modified V regions were cloned into the XbaI sites of the respective plasmid vectors between the cytomegalovirus promoter and the partial introns of the constant regions.

For expression of the chimeric antibody, the heavy chain and kappa chain plasmids were transfected into Sp2/0 mouse myeloma cells by electroporation and cells selected for gpt expression. Clones secreting a maximal amount of complete antibody were detected by ELISA. Purified chimeric CMV5 antibody was shown to bind to CMV-infected cells, which express the gH antigen, by immunostaining of CMV-infected human embryonic lung fibroblasts.

Computer modeling of humanized antibodies.

In order to retain high binding affinity in the humanized antibodies, the general procedures of Queen et al. were followed (see, Queen et al., Proc. Natl. Acad. Sci. USA 86, 10029 (1989) and WO 90/07861). The more homologous a human antibody is to the original murine antibody, the less likely will combining the murine CDR's with the human framework be to introduce distortions into the CDR's that could reduce affinity. Normally the heavy chain and light chain from the same human antibody are chosen to provide the framework sequences, so as to reduce the possibility of incompatibility in the assembling of the two chains. Based on sequence homology search against the NBRF protein sequence database (performed with the MicroGenie Sequence Analysis Software (Beckman)), the antibody Wol was chosen to provide the framework sequences for humanization of CMV5.

The computer program ENCAD (M. Levitt, J. Mol. Biol. 168, 595 (1983)) was used to construct a model of the CMV5 variable region. The model was used to determine the amino acids in the CMV5 framework that were close enough to the CDR's to potentially interact with them (category 4 below). To design the humanized light and heavy chain CMV5 variable regions, at each position the amino acid was chosen to be the same as in the Wol antibody, unless that position fell in one or more of five categories:

(1) The position fell within a CDR,

(2) The Wol amino acid was unusual for human antibodies at that position, whereas the CMV5 amino acid was typical for human antibodies at that position,

(3) The position was immediately adjacent to a CDR,

(4) The model described above suggested that the amino acid may be physically close to the antigen binding region (CDR's).

In category (2), "unusual" is interpreted to include amino acids that occur in less than about 20% of the human sequences

in the same subgroups (as defined by Kabat et al., op. cit.) as the Wol light and heavy chains, and "typical" is interpreted to include amino acids that occur in more than about 25% but generally more than 50% of the human sequences in those subgroups. For positions in these categories, the amino acid from the mouse CMV5 antibody was used. In addition, a position was in the fifth category if the Wol amino acid was highly unusual for human antibodies at that position, and the CMV5 amino acid was different but also unusual. Then an amino acid typical for human antibodies at that position may be used.

The amino acids in each category are shown in Table 1. Some amino acids may be in more than one category. The final sequences of the humanized CMV5 light and heavy chain variable domains are shown in Fig. 27A-B, compared with the Wol sequences.

TABLE 9

Category	Light Chain	Heavy Chain
1	24-34, 50-56, 89-97	31-35, 50-66, 99-108 69, 80
2		69, 80
3	49	30
4		24, 27, 28, 30, 97
5		5

For the construction of genes for the humanized antibodies, nucleotide sequences were selected that encode the protein sequences of the humanized heavy and light chains, including the same signal peptides as in the mouse CMV5 chains (Fig. 25), generally utilizing codons found in the mouse sequence. Several degenerate codons were changed to create restriction sites or to remove undesirable ones. The nucleotide sequences also included the same splice donor signals used in the chimeric genes and an XbaI site at each end. Each gene was constructed from four overlapping

synthetic oligonucleotides. For each variable domain gene, two pairs of overlapping oligonucleotid s on alternating strands were synthesized that encompassed the entire coding sequences as well as the signal peptide and the splice donor signal (Fig. 28). The oligonucleotides were synthesized on an Applied Biosystems 380B DNA synthesizer. Each oligo was about 110-140 bases long with about a 15 base overlap. Double stranded DNA fragments were synthesized with Klenow polymerase from each pair of oligonucleotides, digested with restriction enzymes, ligated to the pUC18 vector and sequenced. Two fragments with the respectively correct half-sequences were then ligated into the XbaI sites of the pVg1-dhfr or pVk expression vectors in the appropriate orientations to produce the complete heavy and light chain genes. Reactions were carried out under conditions well-known in the art (Maniatis et al., op. cit.)

The heavy chain and light chain plasmids are transfected into Sp2/0 mouse myeloma cells by electroporation and cells are selected for gpt expression. Clones are screened by assaying human antibody production in the culture supernatant by ELISA, and antibody purified from the best-producing clones. Antibody is purified by passing tissue culture supernatant over a column of staphylococcal protein A-Sepharose CL-4B (Pharmacia). The bound antibody is eluted with 0.2 M Glycine-HC1, pH3.0 and neutralized with 1 M Tris PH8.0. The buffer is exchanged into PBS by passing over a PD10 column (Pharmacia).

Humanized antibody was also produced by transient transfection. The heavy chain and light chain plasmids were transfected into S194 cells (ATCC TIB 19) by the DEAE-dextran method (Queen et al., Mol. Cell. Biol. 4, 1043 (1984)), and humanized CMV5 antibody was purified from the media supernatant as above. Antibody was quantitated by ELISA assay for human Ig.

Properties of humanized antibodies.

The humanized CMV5 antibody was characterized in comparison to the murine and chimeric antibodies. The humanized CMV5 antibody was shown to bind about as well as the mouse and chimeric antibodies to CMV antigen, by immunostaining of CMV-infected human embryonic lung (HEL) cells (ATCC CCL 137). HEL cells monolayers in 96-well plates were infected wtih CMV at 0.01 pfu/cell, incubated for 4 days, dried at 37°C and stored wrapped at 4°C. 100 µl blotto (5% Carnation Instant Milk in PBS at pH 7.4) was added to each well and incubated at 37°C for 30 min. The blotto was poured off and 75 µl of a series of 2-fold dilutions of mouse, chimeric and humanized CMV5 antibody was added to the wells. The plate was incubated 1 hr at 37°C and washed twice with blotto (each wash was left on for 10 min). Then 75 µl of diluted peroxidase (HRP) conjugated goat anti-mouse or anti-human IgG (Tago) was added to each well and incubated for 1 hr at 37°C. The plate was washed 2x with PBS and 150 µl of HRP substrate solution was added to each well. Color was allowed to develop at room temperature. The plates were washed with water and air dried. The wells were examined under a microscope to determine the highest dilution of the antibodies that formed a colored precipitate on the CMV-infected cells. For all three antibodies, 63 ng/ml was the least amount of antibody that produced a detectable precipitate, indicating that humanized CMV5 binds about as well as the mouse and chimeric antibodies.

To compare the affinities of mouse and humanized CMV5 in another way, a competition experiment was performed. Plates of CMV-infected HEL cells as above were incubated with blotto for 30 min at 37°C. The blotto was poured off and dilutions of mouse or humanized CMV5 were added to each well in 75 µl of PBS. Then 125 µl of radio-iodinated mouse CMV5 (1 µCi/µg) in PBS, containing 28,000 cpm was added to each well and incubated at 37°C for 2.5 hr. The plate was washed 5 times with PBS, and the contents of each well were solubilized with 200 µl of 2% SDS and counted. Increasing concentrations

of mouse and humanized CMV5 inhibited binding of the radiolabeled CMV5 about equally well (Fig. 29), so humanized CMV5 has approximatley the same binding affinity as mouse CV5. An irrelevant antibody did not compete in this assay.

The ability of humanized CMV5 to neutralize CMV is compared to that of mouse CMV5. Mouse and humanized CMV5 are successively diluted by 2-fold in 100 μl of DME medium + 2% FCS in wells of a 96-well plate. 100 μl of CMV, which has been diluted to contain 100 tissue culture infectious dose-50% (TCID50) units, are added to each well and incubated for 60 min at 37°C. Each well of antibody-virus mixture is added to a well of subconfluent HEL cells in a 96-well plate from which the medium has been removed. The cells are incubated for 5 days and cytopathic effect (CPE) is examined in each well under a microscope. The highest dilution of antibody that inhibits CPE by 90% is a measure of the neutralizing ability of the antibody. The humanized CMV5 antibody will neutralize CMV antibody approximately as well as the mouse CMV5 antibody.

Example V

In mammals, the immune response is mediated by several types of cells that interact specifically with foreign material, i.e., antigens. One of these cell types, B cells, is responsible for the production of antibodies. Another cell type, T cells, include a wide variety of cellular subsets that destroy virally infected cells or control the in vivo function of both B cells and other hematopoietic cells, including T cells. A third cell type, macrophages, process and present antigens in conjunction with major histocompatibility complex (MHC) proteins to T cells. Communication between these cell types is mediated in a complex manner by lymphokines, such as interleukins 1-6 and γ-IFN (see, generally, Paul, W.E., ed., Fundamental Immunology, 2nd ed., Raven Press, New York (1989)).

One important lymphokine is γ-IFN, which is secreted by some T cells. In addition to its anti-viral activity, γ-IFN stimulates natual killer (NK) cells, activates

macrophages, and stimulates the expression of MHC molecules on the surface of cells (Paul, op. cit., pp. 622-624). Hence γ-IFN generally serves to enhance many aspects of immune function, and is a logical candidate for a therapeutic drug in cases where such enhancement is desired, e.g., in treating cancer. Conversely, in disease states where the immune system is over-active, e.g., autoimmune diseases and organ transplant rejection, antagonists of γ-IFN may be used to treat the disease by neutralizing the stimulatory effects of γ-IFN.

One class of effective antagonists of γ-IFN are monoclonal antibodies that bind to and neutralize it (see, e.g., Van der Meide et al., J. Gen. Virol, 67, 1059 (1986)). In in vitro and in vivo mouse models of transplants, anti-γ-IFN antibodies have been shown to delay or prevent rejection (Landolfo et al., Science 229, 176 (1985) and Rosenberg et al., J. Immunol. 144, 4648 (1990)). Treatment of mice prone to develop a syndrome like systemic lupus erythematosus (SLE) with a monoclonal antibody to γ-IFN significantly delayed onset of the disease (Jacob et al., J. Exp. Med. 166, 798 (1987)). Under some conditions, an anti-γ-IFN antibody alleviated adjuvant arthritis in rats (Jacob et al., J. Immunol. 142, 1500 (1989)), suggesting that anti-γ-IFN may be effective against some cases of rheumatoid arthritis in human patients. Multiple sclerosis (MS) in patients is made worse by treatment with γ-IFN (Panitch et al., Neurology 36 (suppl. 1), 285 (1986)), so an anti-γ-IFN antibody may alleviate MS. Thus, an anti-γ-IFN antibody may be effective in treating these and other autoimmune diseases.

For treatment of human patients, a murine monoclonal that binds to and neutralizes human γ-IFN (see, e.g., Yamamoto et al., Microbiol. Immunol. 32, 339 (1988)) may be used. Another murine monoclonal antibody designated AF2 that neutralizes human γ-IFN, and inhibits binding of γ-IFN to its cellular receptor, is disclosed herein. Unfortunately, the use of non-human monoclonal antibodies such as AF2 have certain drawbacks in human treatment, particularly in repeated therapeutic regimens.

In accordance with the present invention, humanized immunoglobulins specifically reactive with γ-IFN epitopes are provided. These immunoglobulins, which have binding affinities to γ-IFN of at least about 10^7 M^{-1}, and preferably 10^8 M^{-1} to 10^{10} M^{-1} or stronger, are capable of, e.g., neutralizing human γ-IFN. The humanized immunoglobulins will have a human framework and will have one or more complementarity determining regions (CDR's) from an immunoglobulin, typically a mouse immunoglobulin, specifically reactive with γ-IFN. In a preferred embodiment, one or more of the CDR's will come from the AF2 antibody. Thus, the immunoglobulins of the present invention, which can be produced economically in large quantities, find use, for example, in the treatment of autoimmune disorders in human patients by a variety of techniques.

In one aspect, the present invention is directed to recombinant DNA segments encoding the heavy and/or light chain CDR's from an immunoglobulin capable of binding to a desired epitope of γ-IFN, such as monoclonal antibody AF2. The DNA segments encoding these regions will typically be joined to DNA segments encoding appropriate human framework regions. Exemplary DNA sequences, which on expression code for the polypeptide chains comprising the heavy and light chain CDR's of monoclonal antibody AF2 are included in Fig. 30. Due to codon degeneracy and non-critical amino-acid substitutions, other DNA sequences can be readily substituted for those sequences, as detailed below.

Alternatively, polypeptide fragments comprising only a portion of the primary antibody structure may be produced, which fragments possess one or more immunoglobulin activities (e.g., complement fixation activity). These polypeptide fragments may be produced by proteolytic cleavage of intact antibodies by methods well known in the art, or by inserting stop codons at the desired locations in the vectors pVk and pVg1-dhfr (Fig. 31) using site-directed mutagenesis, such as after CH1 to produce Fab fragments or after the hinge region to produce $(Fab')_2$ fragments. Single chain antibodies may be

produced by joining VL and VH with a DNA linker (see Huston et al., op cit., and Bird et al., op cit.).

The antibodies of the present invention will typically find use individually in treating autoimmune conditions. For example, typical disease states suitable for treatment include graft versus host disease and transplant rejection in patients undergoing an organ transplant, such as heart, lungs, kidneys, liver, etc. Other diseases include autoimmune diseases, such as Type I diabetes, multiple sclerosis, rheumatoid arthritis, systemic lupus erythematosus, and myasthenia gravis.

Humanized immunoglobulins of the present invention may also be used in combination with other antibodies, particularly humanized antibodies reactive with other lymphokines or lymphokine receptors. For example, suitable antigens to which a cocktail of humanized immunoglobulins may react include interleukins 1 through 10 and the p55 and p75 chains of the IL-2 receptor (see, Waldmann, Annu. Rev. Biochem. 58, 875 (1989) and Queen et al., Proc. Natl. Acad. Sci. USA 86, 10029 (1989)). Other antigens include those on cells responsible for the disease, e.g., the so-called "Clusters of Differentiation" (Leucocyte Typing III, ed. by A.J. McMichael, Oxford University Press (1987)).

The antibodies can also be used as separately administered compositions given in conjunction with chemotherapeutic agents. Typically, the agents may include non-steroidal anti-inflammatory agents (e.g., aspirin, ibuprofen), steroids (e.g., prednisone) and immunosuppressants (e.g., cyclosporin A, cytoxan), but numerous additional agents well-known to those skilled in the art may also be utilized. A preferred pharmaceutical composition of the present invention comprises the use of the subject immunoglobulins in immunotoxins, e.g., to kill γ-IFN-secreting cells.

Humanized antibodies of the present invention can further find a wide variety of utilities in vitro. By way of example, the antibodies can be utilized for detection of γ-IFN antigens, or the like.

EXPERIMENTAL

Cloning of heavy chain and light chain cDNA.

cDNAs for the heavy chain and light chain variable domain genes were cloned using anchored polymerase chain reactions (E.Y. Loh et al., Science 243, 217 (1989)), using 3' primers that hybridized to the constant regions and contained HindIII sites, and 5' primers that hybridized to the dG tails and contained EcoR I sites (scheme shown in Fig. 6). The PCR amplified fragments were digested with EcoR I and HindIII and cloned into the pUC18 vector for sequencing. For AF2, two gamma-2b specific and two kappa specific clones were sequenced. The two gamma-2b clones and two kappa clones are respectively identical in sequence. The cDNA variable domain sequences and the deduced amino acid sequences are shown in Fig. 30.

Construction and expression of chimeric antibody.

Two plasmid vectors were prepared for construction and expression of the chimeric antibody genes. The plasmid pVg1-dhfr (Fig. 31A) contains a human cytomegalovirus IE1 promoter and enhancer (M. Boshart et al., Cell 41, 521 (1985)), the human genomic Cγ1 segment including part of the preceding intron, and a dihydrofolate reductase (dhfr) gene (Simonsen et al., Proc. Natl. Acad. Sci. USA 80, 2495 (1984)) for selection. The plasmid pVk (Fig. 31B) is similar to pVg1-dhfr but contains the human genomic Cκ segment and the gpt gene. Derivatives of the AF2 heavy and light chain variable regions were prepared from the cDNAs by polymerase chain reaction. The 5' primers hybridized to the V regions starting at the ATG codons and contained XbaI sites; the 3' primers hybridized to the last 15 nucleotides of the J regions and contained splice donor signals and XbaI sites (see, Queen et al., Proc. Natl. Acad. Sci. USA 86, 10029 (1989)). The modified V regions were cloned into the XbaI sites of th respective plasmid vectors between the CMV promoter and the partial introns of the constant regions.

For expression of the chimeric antibody, the heavy chain and kappa chain plasmids were transfected into Sp2/0 mouse myeloma cells by electroporation and cells selected for gpt expression. Clones secreting a maximal amount of complete antibody were detected by ELISA. Chimeric AF2 antibody was shown to bind to human γ-IFN by ELISA.

Computer modeling of humanized antibodies.

In order to retain high binding affinity in the humanized antibodies, the general procedures of Queen et al. were followed (see, Queen et al., Proc. Natl. Acad. Sci. USA 86, 10029 (1989) and WO 90/07861). The more homologous a human antibody is to the original murine antibody, the less likely will combining the murine CDR's with the human framework be to introduce distortions into the CDR's that could reduce affinity. Normally the heavy chain and light chain from the same human antibody are chosen to provide the framework sequences, so as to reduce the possibility of incompatibility in the assembling of the two chains. Based on sequence homology search against the NBRF protein sequence database (performed with the MicroGenie Sequence Analysis Software (Beckman)), the antibody Eu was chosen to provide the framework sequences for humanization of AF2.

The computer program ENCAD (M. Levitt, J. Mol. Biol. 168, 595 (1983)) was used to construct a model of the AF2 variable region. The model was used to determine the amino acids in the AF2 framework that were close enough to the CDR's to potentially interact with them (category 4 below). To design the humanized light and heavy chain AF2 variable regions, at each position the amino acid was chosen to be the same as in the Eu antibody, unless that position fell in one or more of five categories:

(1) The position fell within a CDR,

(2) The Eu amino acid was unusual for human antibodies at that position, whereas the AF2 amino acid was typical for human antibodies at that position,

(3) The position was immediately adjacent to a CDR,

(4) The model described above suggested that the amino acid may be physically close to the antigen binding region (CDR's).

In category (2), "unusual" is interpreted to include amino acids that occur in less than about 20% of the human sequences in the same subgroups (as defined by Kabat et al., op. cit.) as the Eu light and heavy chains, and "typical" is interpreted to include amino acids that occur in more than about 25% but generally more than 50% of the human sequences in those subgroups. For positions in these categories, the amino acid from the mouse AF2 antibody was used. In addition, a position was in the fifth category if the Eu amino acid was highly unusual for human antibodies at that position, and the AF2 amino acid was different but also unusual. Then an amino acid typical for human antibodies at that position may be used.

The amino acids in each category are shown in Table 10. Some amino acids may be in more than one category. The final sequences of the humanized AF2 light and heavy chain variable domains are shown in Fig. 32, compared with the Eu sequences.

TABLE 10

Category	Light Chain	Heavy Chain
1	24-34, 50-56, 89-97	31-35, 50-66, 99-106
2	48	93, 95, 98, 107, 108, 109, 111
3		30, 98, 107
4	48, 70	27, 28, 30, 98, 107
5	63	

For the construction of genes for the humanized antibodies, nucleotide sequ nces were selected that encode the protein sequences of the humanized heavy and light chains, plus typical immunoglobulin signal sequences, generally

utilizing codons found in the mouse sequence. Several degenerate codons were changed to create restriction sites or to remove undesirable ones. The nucleotide sequences also included the same splice donor signals used in the chimeric genes and an XbaI site at each end. Each gene was constructed from four overlapping synthetic oligonucleotides. For each variable domain gene, two pairs of overlapping oligonucleotides on alternating strands were synthesized that encompassed the entire coding sequences as well as the signal peptide and the splice donor signal (Fig. 33) The oligonucleotides were synthesized on an Applied Biosystems 380B DNA synthesizer. Each oligo was about 110-140 bases long with about a 15 base overlap. Double stranded DNA fragments were synthesized with Klenow polymerase from each pair of oligonucleotides, digested with restriction enzymes, ligated to the pUC18 vector and sequenced. Two fragments with the respectively correct half-sequences are then ligated into the XbaI sites of the pVg1-dhfr or pVk expression vectors in the appropriate orientations to produce the complete heavy and light chain genes. Reactions are carried out under conditions well-known in the art (Maniatis et al., op. cit.)

The heavy chain and light chain plasmids are transfected into Sp2/0 mouse myeloma cells by electroporation and cells selected for gpt expression. Clones are screened by assaying human antibody production in the culture supernatant by ELISA, and antibody purified from the best-producing clones. Antibody is purified by passing tissue culture supernatant over a column of staphylococcal protein A-Sepharose CL-4B (Pharmacia). The bound antibody is eluted with 0.2 M Glycine-HCl, pH3.0 and neutralized with 1 M Tris PH8.0. The buffer is exchanged into PBS by passing over a PD10 column (Pharmacia).

Properties of humanized antibodies.

The humanized AF2 antibody is characterized in comparison to the murine and chimeric antibodies. The humanized antibody will bind to γ-IFN in an ELISA assay in a

manner similar to the mouse and chimeric antibodies, showing that it recognizes γ-IFN.

To compare the binding affinities of mouse AF2 antibody and humanized AF2 antibody, a competitive ELISA assay is performed. An ELISA plate is coated with human recombinant γ-IFN by adding 100 μl of a 500 ng/ml solution of γ-IFN in PBS to each well and incubating overnight at 4°C. Subsequent steps are carried out at room temperature. The γ-IFN solution is removed and 200 μl of ELISA buffer (0.1% Tween-20, 1% Bovine serum albumin in PBS) is added to each well and incubated for 1 hr. After removing the solution, varying amounts of competitor antibody (mouse AF2 or humanized AF2) in 100 μl PBS is added to each well, along with an amount of biotinylated AF2 predetermined to give a good ELISA response. The plate is incubated for 1 hr and then washed 3 times with ELISA buffer. An amount of horseradish peroxidase (HRP)-conjugated strepavidin predetermined to be in excess is added in 100 μl PBS to each well and incubated for 30 min. The plate is washed 3 times in ELISA buffer, and 100 μl of substrate solution for HRP is added to each well. The plate is incubated for 10-30 min, and the optical density of each well is determined with an ELISA reader (BioRad). The decrease in optical density with increasing concentrations of competitor antibodies mouse AF2 and humanized AF2 are plotted. Mouse AF2 and humanized AF2 will compete similarly, showing that their binding affinities for γ-IFN are approximately the same. The procedures used are well known in the art (e.g., Harlow and Lane, op. cit.).

An important biological activity of γ-IFN is the induction of expression of class II HLA antigens on cells. To determine the ability of mouse and humanized AF2 to neutralize this activity, about 5 x 10^4 HS294T cells (Basham et al., J. Immunol. 130, 1492 (1983)) are plated in 1.0 ml DMEM medium + 10% FCS in each well of a 24-well plate. After overnight incubation, 0.1 nM interferon and varying amounts of mouse or humanized AF2 are added to the cells, and the plate is incubated for 72 hr. The cells are removed from the plate

with 0.05 M EDTA, stained with monoclonal antibody L243 from the American Type Culture Collection (ATCC) against HLA-D antigen, washed, stained with FITC conjugated goat anti-mouse Ig and analyzed with a FACScan (Becton-Dickinson). Increasing concentrations of mouse AF2 reduce fluorescence of the cells (Fig. 34), indicating the antibody is preventing induction of HLA-D by γ-IFN. The humanized AF2 will act similarly to mouse AF2 in this assay, showing that it neutralizes the biological activity of γ-IFN.

From the foregoing, it will be appreciated that the humanized immunoglobulins of the present invention offer numerous advantages over other γ-IFN specific antibodies. In comparison to mouse monoclonal antibodies, the present humanized immunoglobulins can be more economically produced and contain substantially less foreign amino acid sequences. This reduced likelihood of antigenicity after injection into a human patient represents a significant therapeutic improvement.

All publications and patent applications are herein incorporated by reference to the same extent as if each individual publication or patent application was specifically and individually indicated to be incorporated by reference. Although the present invention has been described in some detail by way of illustration and example for purposes of clarity and understanding, it will be apparent that certain changes and modifications may be practiced within the scope of the appended claims.

WHAT IS CLAIMED IS

1. A composition comprising a substantially pure humanized immunoglobulin specifically reactive with the p75 chain of the human IL-2 receptor.

2. A composition according to Claim 1, wherein the immunoglobulin exhibits a binding affinity to a human IL-2 receptor of about 10^7 M^{-1} or stronger.

3. A composition according to Claim 1, wherein the immunoglobulin comprises one or more foreign CDRs substantially homologous to a CDR from an immunoglobulin reactive with human p75 protein.

4. A composition according to Claim 1, wherein the immunoglobulin is capable of blocking the binding of interleukin-2 (IL-2) to the p75 chain of human IL-2 receptors.

5. A composition according to Claim 1, wherein the humanized immunoglobulin comprises the human framework regions having amino acids sequences from at least two human immunoglobulins.

6. A humanized immunoglobulin capable of binding to human interleukin-2 receptors, said immunoglobulin comprising one or more complementarity determining regions (CDR's) from mik-β1 antibody in a human framework.

7. A humanized immunoglobulin according to Claim 6, wherein the human framework is substantially homologous to an Lay immunoglobulin framework.

8. A humanized immunoglobulin according to Claim 6 which is capable of blocking the binding of IL-2 to interleukin-2 receptors on human T-cells.

9. A method of treating T-cell mediated disorders in a human patient, said method comprising administering to said patient a therapeutically effective dose of an immunoglobulin according to Claim 6.

10. A humanized immunoglobulin according to Claim 6 which is complexed to a cytotoxic agent.

11. A composition comprising a substantially pure humanized immunoglobulin specifically reactive with a herpes simplex virus-specific epitope.

12. A composition according to claim 11, wherein the epitope is on a viral surface glycoprotein.

13. A composition according to claim 12, wherein the glycoprotein is gB or gD.

14. A composition comprising a substantially pure humanized immunoglobulin capable of inhibiting binding of a herpes simplex virus (HSV) protein to a mouse monoclonal antibody specifically reactive with said protein, wherein the humanized immunoglobulin comprises at least one complementarity determining region (CDR) from the mouse monoclonal antibody.

15. A composition according to Claim 14, wherein the humanized immunoglobulin exhibits a binding affinity of about 10^7 M^{-1} or stronger.

16. A composition according to Claim 14 wherein said immunoglobulin is capable of binding to type 1 or 2 herpes simplex virus (HSV).

17. A composition according to Claim 14, wherein the immunoglobulin comprises one or more CDR's substantially

homologous to a CDR from an immunoglobulin reactive with HSV glycoprotein of gB, gD, gG or gH.

18. A composition according to Claim 14, wherein the immunoglobulin is an IgG_1 immunoglobulin isotype.

19. A humanized immunoglobulin capable of binding to herpes simplex virus, said immunoglobulin comprising one or more complementarity determining regions (CDR's) from a mouse monoclonal antibody in a human framework, wherein the mouse antibody is Fd 79 or Fd 138-80.

20. A humanized immunoglobulin according to Claim 19, wherein the human framework is substantially homologous to an Eu or a Pom immunoglobulin framework.

21. A humanized immunoglobulin according to Claim 19 which is capable of neutralizing HSV.

22. A method of treating herpes simplex virus mediated disorders in a human patient, said method comprising administering to said patient a therapeutically effective dose of an immunoglobulin according to Claim 14.

23. A composition comprising a substantially pure humanized immunoglobulin specifically reactive with a CD33 antigen epitope.

24. A composition according to Claim 23, wherein a variable region of at least one chain of the immunoglobulin comprises three complementarity determining regions (CDR's) from a non-human antibody in a human framework.

25. A composition according to claim 24, wherein the chain is the heavy chain.

26. A composition according to claim 24, wherein th non-human antibody is M195.

27. A composition comprising a substantially pure humanized immunoglobulin capable of inhibiting binding of CD33 antigen to a mouse monoclonal antibody specifically reactive with said antigen, wherein the humanized immunoglobulin comprises at least one complementarity determining region (CDR) from the mouse monoclonal antibody.

28. A composition according to Claim 27, wherein the humanized immunoglobulin exhibits a binding affinity of about 10^7 M^{-1} or stronger.

29. A composition according to Claim 27, which is capable of blocking the binding of mouse M195 antibody to human cells.

30. A composition according to Claim 27, wherein the humanized immunoglobulin comprises a human framework substantially homologous to Eu immunoglobulin framework.

31. A humanized immunoglobulin according to Claim 27 which is of capable mediating antibody-dependent cellular cytotoxicity in the presence of human target and effector cells.

32. A method of treating myeloid cell-mediated disorders in a human patient, said method comprising administering to said patient a therapeutically effective dose of a composition according to Claim 27.

33. A composition according to claim 27, wherein the immunoglobulin is conjugated to a cytoxic agent.

34. A composition comprising a substantially pure humanized immunoglobulin specifically reactive with a human cytomegalovirus-specific epitope.

35. A composition according to Claim 34, wherein a variable region of at least one chain comprises three complementarity determining regions from a non-human immunoglobulin chain in a human framework.

36. A composition according to claim 34, wherein the epitope is on a viral surface glycoprotein.

37. A composition according to claim 36, wherein the glycoprotein is gB or gH.

38. A composition comprising a substantially pure humanized immunoglobulin capable of inhibiting binding of a cytomegalovirus (CMV) protein to a mouse monoclonal antibody specifically reactive with said protein, wherein the humanized immunoglobulin comprises at least one complementarity determining region (CDR) from the mouse monoclonal antibody.

39. A composition according to Claim 38, wherein the humanized immunoglobulin exhibits a binding affinity of about 10^7 M^{-1} or stronger.

40. A recombinant immunoglobulin composition comprising a human framework and one or more foreign complementarity determining regions (CDR's) not naturally associated with the framework, wherein said immunoglobulin is capable of binding to CMV.

41. A composition according to Claim 40, wherein all of the foreign CDR's are located on heavy chains of the immunoglobulin.

42. A composition according to Claim 40, wherein the immunoglobulin is an IgG_1 immunoglobulin isotype.

43. A composition according to Claim 40 wherein the immunoglobulin is capable of blocking the binding of CMV to human cells.

44. An immunoglobulin according to Claim 40, wherein the framework regions comprise amino acids sequences from at least two human immunoglobulins.

45. A humanized immunoglobulin capable of binding to cytomegalovirus, said immunoglobulin comprising one or more complementarity determining regions (CDR's) from a mouse monoclonal antibody in a human framework, wherein the mouse antibody is CMV5, CMV109 or CMV115.

46. A humanized immunoglobulin according to Claim 45, wherein the human framework is substantially homologous to an Eu or a Wol immunoglobulin framework.

47. A humanized immunoglobulin according to Claim 45 which is capable of neutralizing CMV.

48. A method of treating cytomegalovirus mediated disorders in a human patient, said method comprising administering to said patient a therapeutically effective dose of an immunoglobulin according to Claim 45.

49. A method of treating cytomegalovirus mediated disorders in a human patient, said method comprising administering to said patient a therapeutically effective dose of a combination of two or more immunoglobulins according to Claims 45.

50. A composition comprising a substantially pure humanized immunoglobulin specifically reactive with human γ-IFN.

51. A composition according to Claim 50, wherein a variable region of at least one chain comprises three complementarity determining regions (CDR's) from a non-human antibody in a human framework.

52. A composition according to claim 51, wherein the non-human antibody is AF2.

53. A composition according to Claim 50 capable of inhibiting binding of human γ-IFN to a human γ-IFN receptor.

54. A recombinant immunoglobulin composition comprising a human framework and one or more complementarity determining regions (CDR's) not naturally associated with the framework, wherein said immunoglobulin is capable of specifically inhibiting biniding of human γ-IFN to a human γ-IFN receptor.

55. A composition according to Claim 54, wherein one or more of the foreign CDR's are substantially homologous to a CDR from the AF2 antibody.

56. A composition according to Claim 54, wherein the immunoglobulin is an IgG_1 immunoglobulin isotype.

57. A composition according to Claim 54, wherein the immunoglobulin is capable of blocking the binding of human γ-IFN antibody to human cells.

58. A method of treating autoimmune disorders in a human patient, said method comprising administering to said patient a therapeutically effective dose of a composition according to Claim 54.

59. A composition according to Claim 11, wherein the immunoglobulin is conjugated to a cytotoxic agent.

60. A composition according to Claim 40, wherein the immunoglobulin is conjugated to a cytotoxic agent.

1	D	I	V	L	T	Q	S	P	A	S	L	A	V	S	L	G	Q	R	A	T
1	E	I	V	M	T	Q	S	P	A	T	L	S	V	S	P	G	E	R	A	T
21	I	S	C	R	A	S	Q	S	V	S	T	S	T	Y	N	Y	M	H	W	Y
21	L	S	C	R	A	S	Q	S	V	S	T	S	T	Y	N	Y	M	H	W	Y
41	Q	Q	K	P	G	Q	P	P	K	L	L	I	K	Y	A	S	N	L	E	S
41	Q	Q	K	P	G	Q	S	P	R	L	L	I	K	Y	A	S	N	L	E	S
61	G	V	P	A	R	F	S	G	S	G	F	G	T	D	F	T	L	N	I	H
61	G	I	P	A	R	F	S	G	S	G	S	G	T	E	F	T	L	T	I	S
81	P	V	E	E	E	D	T	V	T	Y	Y	C	Q	H	S	W	E	I	P	Y
81	R	L	E	S	E	D	F	A	V	Y	Y	C	Q	H	S	W	E	I	P	Y
101	T	F	G	G	G	T	K	L	E	I	K									
101	T	F	G	Q	G	T	R	V	E	I	K									

FIG. 1A

1	E	M	I	L	V	E	S	G	G	G	L	V	K	P	G	A	S	L	K	L
1	E	V	Q	L	L	E	S	G	G	G	L	V	Q	P	G	G	S	L	R	L
21	S	C	A	A	S	G	F	T	F	S	N	Y	G	L	S	W	V	R	Q	T
21	S	C	A	A	S	G	F	T	F	S	N	Y	G	L	S	W	V	R	Q	A
41	S	D	R	R	L	E	W	V	A	S	I	S	R	G	G	G	R	I	Y	S
41	P	G	K	G	L	E	W	V	A	S	I	S	R	G	G	G	R	I	Y	S
61	P	D	N	L	K	G	R	F	T	I	S	R	E	D	A	K	N	T	L	Y
61	P	D	N	L	K	G	R	F	T	I	S	R	N	D	S	K	N	T	L	Y
81	L	Q	M	S	S	L	K	S	E	D	T	A	L	Y	Y	C	L	R	E	G
81	L	Q	M	N	S	L	Q	A	E	D	T	A	L	Y	Y	C	L	R	E	G
101	I	Y	Y	A	D	Y	G	F	F	D	V	W	G	T	G	T	T	V	I	V
101	I	Y	Y	A	D	Y	G	F	F	D	V	W	G	Q	G	T	L	V	T	V
121	S	S																		
121	S	S																		

FIG. 1B

1	D	I	V	M	T	Q	S	H	K	F	M	S	T	S	V	G	D	R	V	S
1	D	I	Q	M	T	Q	S	P	S	T	L	S	A	S	V	G	D	R	V	T
21	I	T	C	K	A	S	Q	D	V	G	S	A	V	V	W	H	Q	Q	K	S
21	I	T	C	K	A	S	Q	D	V	G	S	A	V	V	W	H	Q	Q	K	P
41	G	Q	S	P	K	L	L	I	Y	W	A	S	T	R	H	T	G	V	P	D
41	G	K	A	P	K	L	L	I	Y	W	A	S	T	R	H	T	G	V	P	S
61	R	F	T	G	S	G	S	G	T	D	F	T	L	T	I	T	N	V	Q	S
61	R	F	T	G	S	G	S	G	T	E	F	T	L	T	I	S	S	L	Q	P
81	E	D	L	A	D	Y	F	C	Q	Q	Y	S	I	F	P	L	T	F	G	A
81	D	D	F	A	T	Y	F	C	Q	Q	Y	S	I	F	P	L	T	F	G	Q
101	G	T	R	L	E	L	K													
101	G	T	K	V	E	V	K													

FIG. 2A

1	Q	V	Q	L	Q	Q	S	D	A	E	L	V	K	P	G	A	S	V	K	I
1	Q	V	Q	L	V	Q	S	G	A	E	V	K	K	P	G	S	S	V	K	V
21	S	C	K	V	S	G	Y	T	F	T	D	H	T	I	H	W	M	K	Q	R
21	S	C	K	A	S	G	Y	T	F	T	D	H	T	I	H	W	M	R	Q	A
41	P	E	Q	G	L	E	W	F	G	Y	I	Y	P	R	D	G	H	T	R	Y
41	P	G	Q	G	L	E	W	F	G	Y	I	Y	P	R	D	G	H	T	R	Y
61	S	E	K	F	K	G	K	A	T	L	T	A	D	K	S	A	S	T	A	Y
61	A	E	K	F	K	G	K	A	T	I	T	A	D	E	S	T	N	T	A	Y
81	M	H	L	N	S	L	T	S	E	D	S	A	V	Y	F	C	A	R	G	R
81	M	E	L	S	S	L	R	S	E	D	T	A	V	Y	F	C	A	R	G	R
101	D	S	R	E	R	N	G	F	A	Y	W	G	Q	G	T	L	V	T	V	S
101	D	S	R	E	R	N	G	F	A	Y	W	G	Q	G	T	L	V	T	V	S
121	A																			
121	S																			

FIG. 2B

1	D	I	V	L	T	Q	S	P	A	S	L	A	V	S	L	G	Q	R	A	T
1	D	I	Q	M	T	Q	S	P	S	S	L	S	A	S	V	G	D	R	V	T
21	I	S	C	R	A	S	E	S	V	D	N	Y	G	I	S	F	M	N	W	F
21	I	T	C	R	A	S	E	S	V	D	N	Y	G	I	S	F	M	N	W	F
41	Q	Q	K	P	G	Q	P	P	K	L	L	I	Y	A	A	S	N	Q	G	S
41	Q	Q	K	P	G	K	A	P	K	L	L	I	Y	A	A	S	N	Q	G	S
61	G	V	P	A	R	F	S	G	S	G	S	G	T	D	F	S	L	N	I	H
61	G	V	P	S	R	F	S	G	S	G	S	G	T	D	F	T	L	N	I	S
81	P	M	E	E	D	D	T	A	M	Y	F	C	Q	Q	S	K	E	V	P	W
81	S	L	Q	P	D	D	F	A	T	Y	Y	C	Q	Q	S	K	E	V	P	W
101	T	F	G	G	G	T	K	L	E	I	K									
101	T	F	G	Q	G	T	K	V	E	I	K									

FIG. 3A

1	E	V	Q	L	Q	Q	S	G	P	E	L	V	K	P	G	A	S	V	K	I
1	Q	V	Q	L	V	Q	S	G	A	E	V	K	K	P	G	S	S	V	K	V
21	S	C	K	A	S	G	Y	T	F	T	D	Y	N	M	H	W	V	K	Q	S
21	S	C	K	A	S	G	Y	T	F	T	D	Y	N	M	H	W	V	R	Q	A
41	H	G	K	S	L	E	W	I	G	Y	I	Y	P	Y	N	G	G	T	G	Y
41	P	G	Q	G	L	E	W	I	G	Y	I	Y	P	Y	N	G	G	T	G	Y
61	N	Q	K	F	K	S	K	A	T	L	T	V	D	N	S	S	S	T	A	Y
61	N	Q	K	F	K	S	K	A	T	I	T	A	D	E	S	T	N	T	A	Y
81	M	D	V	R	S	L	T	S	E	D	S	A	V	Y	Y	C	A	R	G	R
81	M	E	L	S	S	L	R	S	E	D	T	A	V	Y	Y	C	A	R	G	R
101	P	A	M	D	Y	W	G	Q	G	T	S	V	T	V	S	S				
101	P	A	M	D	Y	W	G	Q	G	T	L	V	T	V	S	S				

FIG. 3B

1	Q	I	V	L	T	Q	S	P	A	I	M	S	A	S	P	G	E	K	V	T
1	D	I	Q	M	T	Q	S	P	S	S	L	S	A	S	V	G	D	R	V	T
21	M	T	C	S	G	S	S	S	V	S	F	M	Y	W	Y	Q	Q	R	P	G
21	I	T	C	S	G	S	S	S	V	S	F	M	Y	W	Y	Q	Q	K	P	G
41	S	S	P	R	L	L	I	Y	D	T	S	N	L	A	S	G	V	P	V	R
41	K	A	P	K	L	L	I	Y	D	T	S	N	L	A	S	G	V	P	S	R
61	F	S	G	S	G	S	G	T	S	Y	S	L	T	I	S	R	M	E	A	E
61	F	S	G	S	G	S	G	T	D	Y	T	F	T	I	S	S	L	Q	P	E
81	D	A	A	T	Y	Y	C	Q	Q	W	S	T	Y	P	L	T	F	G	A	G
81	D	I	A	T	Y	Y	C	Q	Q	W	S	T	Y	P	L	T	F	G	Q	G
101	T	K	L	E	L	K														
101	T	K	V	E	V	K														

FIG. 4A

1	Q	V	Q	L	K	Q	S	G	P	G	L	V	Q	P	S	Q	S	L	S	I
1	E	V	Q	L	L	E	S	G	G	G	L	V	Q	P	G	Q	S	L	R	L
21	T	C	T	V	S	G	F	S	V	T	S	Y	G	V	H	W	I	R	Q	S
21	S	C	A	A	S	G	F	T	V	T	S	Y	G	V	H	W	V	R	Q	A
41	P	G	K	G	L	E	W	L	G	V	I	W	S	G	G	S	T	D	Y	N
41	P	G	K	G	L	E	W	V	G	V	I	W	S	G	G	S	T	D	Y	N
61	A	A	F	I	S	R	L	T	I	S	K	D	N	S	K	S	Q	V	F	F
61	A	A	F	I	S	R	F	T	I	S	R	D	N	S	K	N	T	L	Y	L
81	K	V	N	S	L	Q	P	A	D	T	A	I	Y	Y	C	A	R	A	G	D
81	Q	M	N	S	L	Q	A	E	D	T	A	I	Y	Y	C	A	R	A	G	D
101	Y	N	Y	D	G	F	A	Y	W	G	Q	G	T	L	V	T	V	S	A	
101	Y	N	Y	D	G	F	A	Y	W	G	Q	G	T	L	V	T	V	S	S	

FIG. 4B

1	D	I	V	L	T	Q	S	P	A	T	L	S	V	T	P	G	D	S	V	S
1	E	I	V	L	T	Q	S	P	G	T	L	S	L	S	P	G	E	R	A	T
21	L	S	C	R	A	S	Q	S	I	S	N	N	L	H	W	Y	Q	Q	K	S
21	L	S	C	R	A	S	Q	S	I	S	N	N	L	H	W	Y	Q	Q	K	P
41	H	E	S	P	R	L	L	I	K	Y	A	S	Q	S	I	S	G	I	P	S
41	G	Q	A	P	R	L	L	I	K	Y	A	S	Q	S	I	S	G	I	P	D
61	R	F	S	G	S	G	S	G	T	D	F	T	L	S	V	N	G	V	E	T
61	R	F	S	G	S	G	S	G	T	D	F	T	L	T	I	S	R	L	E	P
81	E	D	F	G	M	Y	F	C	Q	Q	S	N	S	W	P	H	T	F	G	G
81	E	D	F	A	V	Y	Y	C	Q	Q	S	N	S	W	P	H	T	F	G	Q
101	G	T	K	L	E	I	K													
101	G	T	K	V	E	I	K													

FIG. 5A

1	E	V	Q	L	Q	Q	S	G	P	E	L	V	K	P	G	A	S	M	K	I
1	Q	V	Q	L	V	Q	S	G	A	E	V	K	K	P	G	S	S	V	R	V
21	S	C	K	A	S	V	Y	S	F	T	G	Y	T	M	N	W	V	K	Q	S
21	S	C	K	A	S	G	Y	S	F	T	G	Y	T	M	N	W	V	R	Q	A
41	H	G	Q	N	L	E	W	I	G	L	I	N	P	Y	N	G	G	T	S	Y
41	P	G	K	G	L	E	W	V	G	L	I	N	P	Y	N	G	G	T	S	Y
61	N	Q	K	F	K	G	K	A	T	L	T	V	D	K	S	S	N	T	A	Y
61	N	Q	K	F	K	G	R	V	T	V	S	L	K	P	S	F	N	Q	A	Y
81	M	E	L	L	S	L	T	S	A	D	S	A	V	Y	Y	C	T	R	R	G
81	M	E	L	S	S	L	F	S	E	D	T	A	V	Y	Y	C	T	R	R	G
101	F	R	D	Y	S	M	D	Y	W	G	Q	G	T	S	V	T	V	S	S	
101	F	R	D	Y	S	M	D	Y	W	G	Q	G	T	L	V	T	V	S	S	

FIG. 5B

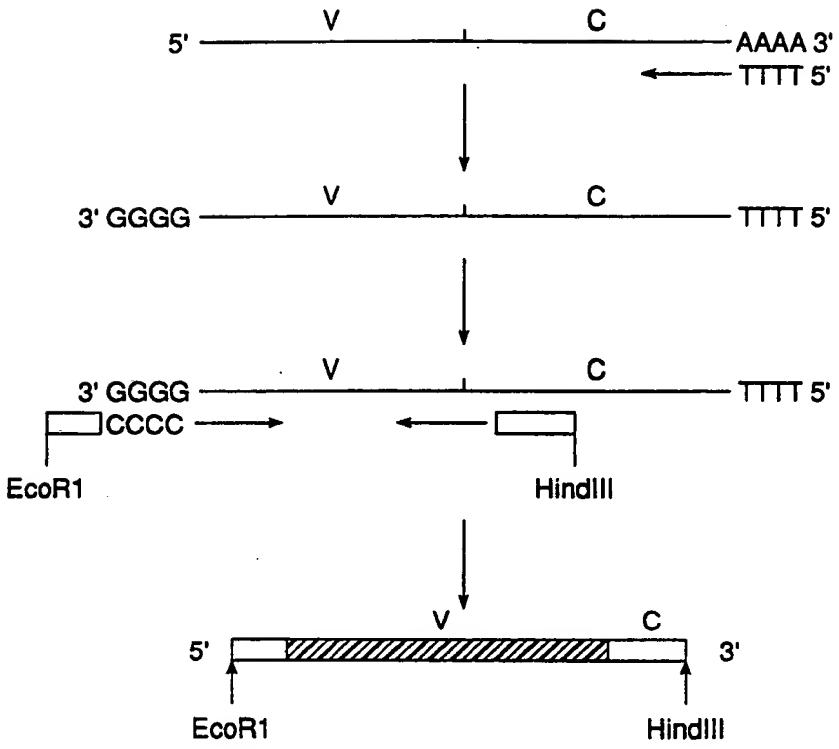

FIG. 6

```
          .         .        30         .         .        60
ATGGATTTTCAAGTGCAGATTTTCAGCTTCCTGCTAATCAGTGCCTCAGTCATACTGTCC
 M  D  F  Q  V  Q  I  F  S  F  L  L  I  S  A  S  V  I  L  S

          .         .        90         .         .       120
AGAGGACAAATTGTTCTCACCCAGTCTCCAGCAATCATGTCTGCGTCTCCAGGGGCGAAG
 R  G  Q  I  V  L  T  Q  S  P  A  I  M  S  A  S  P  G  E  K

          .         .       150         .         .       180
GTCACCATGACCTGCAGTGGCAGCTCAAGTGTAAGTTTCATGTACTGGTACCAGCAGAGG
 V  T  M  T  C  S  G  S  S  S  V  S  F  M  Y  W  Y  Q  Q  R

          .         .       210         .         .       240
CCAGGATCCTCCCCCAGACTCCTGATTTATGACACATCCAACCTGGCTTCTGGAGTCCCT
 P  G  S  S  P  R  L  L  I  Y  D  T  S  N  L  A  S  G  V  P

          .         .       270         .         .       300
GTTCGCTTCAGTGGCAGTGGGTCTGGGACCTCTTACTCTCTCACAATCAGCCGAATGGAG
 V  R  F  S  G  S  G  S  G  T  S  Y  S  L  T  I  S  R  M  E

          .         .       330         .         .       360
GCTGAAGATGCTGCCACTTATTACTGCCAGCAGTGGAGTACTTACCCGCTCACGTTCGGT
 A  E  D  A  A  T  Y  Y  C  Q  Q  W  S  T  Y  P  L  T  F  G

          .         .
GCTGGGACCAAGCTGGAGCTGAAA
 A  G  T  K  L  E  L  K
```

FIG. 7A

```
          .         .        30         .         .        60
ATGGCTGTCTTGGGGCTGCTCTTCTGCCTGGTGACATTCCCAAGCTGTGTCCTATCCCAG
 M  A  V  L  G  L  L  F  C  L  V  T  F  P  S  C  V  L  S  Q

          .         .        90         .         .       120
GTGCAGCTGAAGCAGTCAGGACCTGGCCTAGTGCAGCCCTCACAGAGCCTGTCCATCACC
 V  Q  L  K  Q  S  G  P  G  L  V  Q  P  S  Q  S  L  S  I  T

          .         .       150         .         .       180
TGCACAGTCTCTGGTTTCTCAGTAACAAGTTATGGTGTACACTGGATTCGCCAGTCTCCA
 C  T  V  S  G  F  S  V  T  S  Y  G  V  H  W  I  R  Q  S  P

          .         .       210         .         .       240
GGAAAGGGTCTGGAGTGGCTGGGAGTGATATGGAGTGGTGGAAGCACAGACTATAATGCA
 G  K  G  L  E  W  L  G  V  I  W  S  G  G  S  T  D  Y  N  A

          .         .       270         .         .       300
GCTTTCATATCCAGACTGACCATCAGCAAGGACAACTCCAAGAGCCAAGTTTTCTTTAAA
 A  F  I  S  R  L  T  I  S  K  D  N  S  K  S  Q  V  F  F  K

          .         .       330         .         .       360
GTGAACAGTCTGCAACCTGCTGACACAGCCATATACTATTGTGCCAGAGCTGGGGACTAT
 V  N  S  L  Q  P  A  D  T  A  I  Y  Y  C  A  R  A  G  D  Y

          .         .       390         .         .
AATTACGACGGTTTTGCTTACTGGGGCCAAGGGACTCTGGTCACTGTCTCTGCG
 N  Y  D  G  F  A  Y  W  G  Q  G  T  L  V  T  V  S  A
```

FIG. 7B

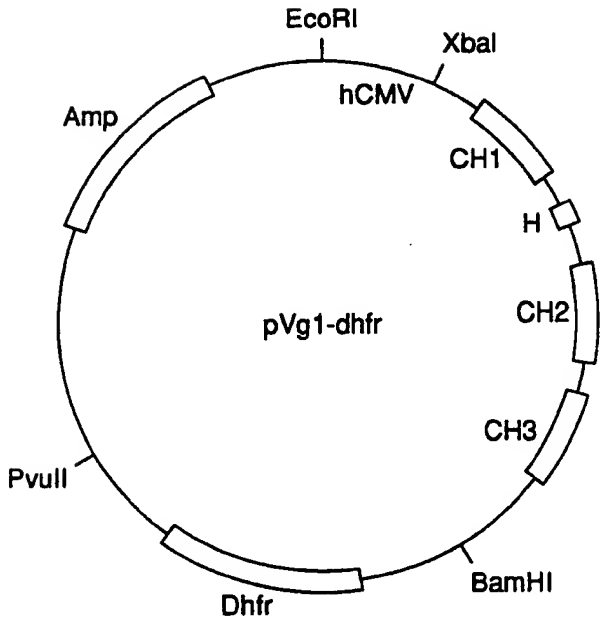
EcoRI
XbaI
hCMV
Amp
CH1
H
pVg1-dhfr
CH2
CH3
PvuII
BamHI
Dhfr

FIG. 8A

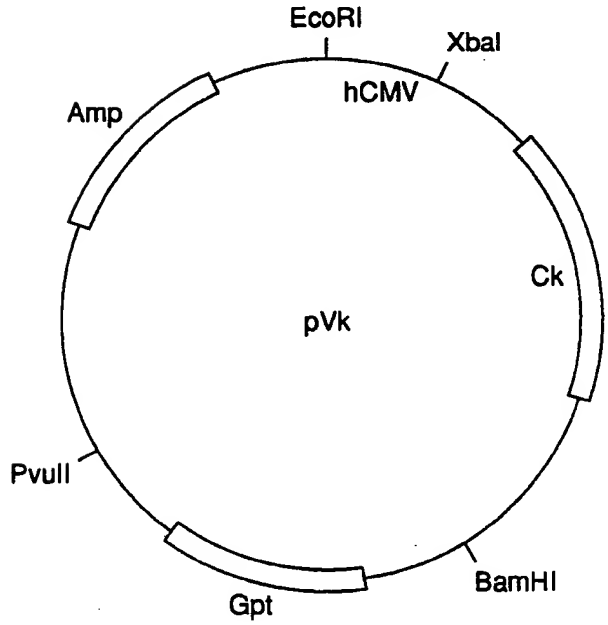
EcoRI
XbaI
hCMV
Amp
Ck
pVk
PvuII
BamHI
Gpt

FIG. 8B

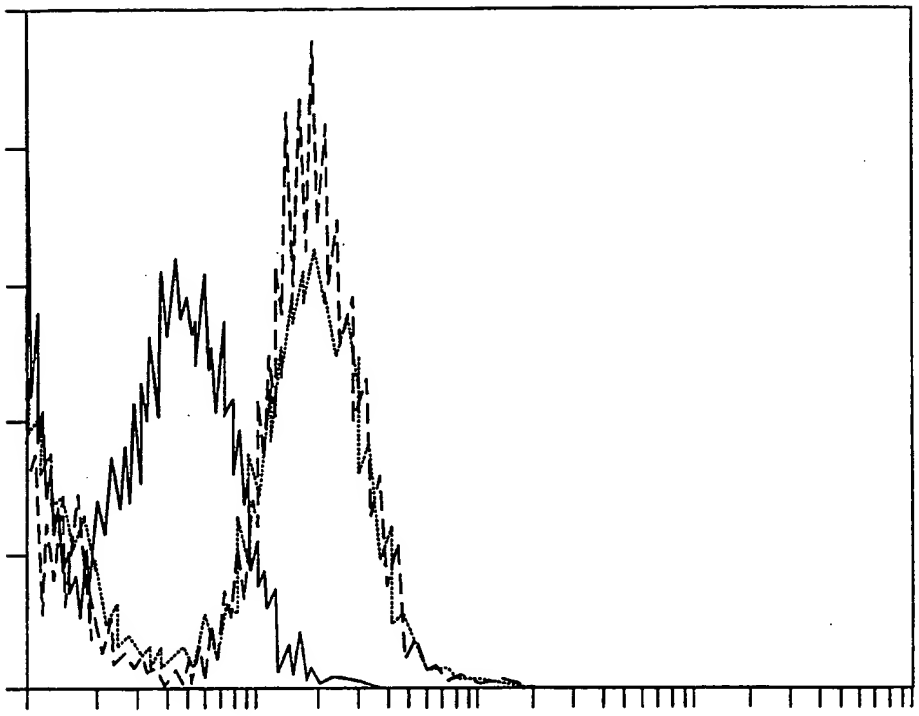

FIG. 9

1	D	I	Q	M	T	Q	S	P	S	S	L	S	V	S	V	G	D	R	V	T
1	D	I	Q	M	T	Q	S	P	S	S	L	S	A	S	V	G	D	R	V	T
21	I	T	C	Q	A	S	Q	N	V	N	A	Y	L	N	W	Y	Q	Q	K	P
21	I	T	C	S	G	S	S	S	V		S	F	M	Y	W	Y	Q	Q	K	P
41	G	L	A	P	K	L	L	I	Y	G	A	S	T	R	E	A	G	V	P	S
40	G	K	A	P	K	L	L	I	Y	D	T	S	N	L	A	S	G	V	P	S
61	R	F	S	G	S	G	S	G	T	D	F	T	F	T	I	S	S	L	Q	P
60	R	F	S	G	S	G	S	G	T	D	Y	T	F	T	I	S	S	L	Q	P
81	E	D	I	A	T	Y	Y	C	Q	Q	Y	N	N	W	P	P	T	F	G	Q
80	E	D	I	A	T	Y	Y	C	Q	Q	W	S	T	Y	P	L	T	F	G	Q
101	G	T	K	V	E	V	K													
100	G	T	K	V	E	V	K													

FIG. 10A

1	A	V	Q	L	L	E	S	G	G	G	L	V	Q	P	G	G	S	L	R	L
1	E	V	Q	L	L	E	S	G	G	G	L	V	Q	P	G	G	S	L	R	L
21	S	C	A	A	S	G	F	T	F	S	A	S	A	M	S	W	V	R	Q	A
21	S	C	A	A	S	G	F	T	V	T	S	Y	G	V	H	W	V	R	Q	A
41	P	G	K	G	L	E	W	V	A	W	K	Y	E	N	G	N	D	K	H	Y
41	P	G	K	G	L	E	W	V	G		V	I	W	S	G	G	S	T	D	Y
61	A	D	S	V	N	G	R	F	T	I	S	R	N	D	S	K	N	T	L	Y
60	N	A	A	F	I	S	R	F	T	I	S	R	D	N	S	K	N	T	L	Y
81	L	Q	M	N	G	L	Q	A	Z	V	S	A	I	Y	Y	C	A	R	D	A
80	L	Q	M	N	S	L	Q	A	E	D	T	A	I	Y	Y	C	A	R		A
101	G	P	Y	V	S	P	T	F	F	A	H	W	G	Q	G	T	L	V	T	V
99	G	D	Y		N	Y	D	G	F	A	Y	W	G	Q	G	T	L	V	T	V
121	S	S																		
118	S	S																		

FIG. 10B

vc13

```
        10          20          30          40          50          60
TTCTGCTGGT ACCAGTACAT GAAACTTACA CTTGAGCTGC CACTGCAGGT GATGGTGACG

        70          80          90         100
CGGTCACCCA CTGAGGCACT GAGGCTAGAT GGAGACTGGG TCATTTG
```

vc14

```
        10          20          30          40          50          60
CATGTACTGG TACCAGCAGA AGCCAGGAAA AGCTCCGAAA CTTCTGATTT ATGACACATC

        70          80          90         100         110         120
CAACCTGGCT TCTGGAGTCC CTTCCCGCTT CAGTGGCAGT GGGTCTGGGA CCGATTACAC

       130
CTTTACAATC TCTTCA
```

vc15

```
        10          20          30          40          50          60
TGTGTCTAGA AAAGTGTACT TACGTTTTAC CTCGACCTTG GTCCCTTGAC CGAACGTGAG

        70          80          90         100         110         120
CGGGTAAGTA CTCCACTGCT GGCAGTAATA AGTGGCTATA TCTTCCGGCT GAAGTGAAGA

       130
GATTGTAAAG GTGTAAT
```

vc16

```
        10          20          30          40          50          60
CACATCTAGA CCACCATGGA TTTTCAAGTG CAGATCTTCA GCTTCCTGCT AATCAGTGCC

        70          80          90         100
TCAGTCATAC TGTCCAGAGG AGATATTCAA ATGACCCAGT CTCCATCT
```

FIG. 11A

vc11

```
        10          20          30          40          50          60
TAGTCTGTCG ACCCACCACT CCATATCACT CCCACCCACT CGAGTCCCTT TCCAGGAGCC

        70          80          90         100         110         120
TGGCGGACCC AGTGTACACC ATAACTTGTT ACGGTGAAAC CACTGGCGGC ACAAGACAGT

       130
CTCAGAGATC CTCCTGGC
```

vc12

```
        10          20          30          40          50          60
TGGTGGGTCG ACAGACTATA ATGCAGCTTT CATATCCAGA TTTACCATCA GCAGAGACAA

        70          80          90         100         110         120
CAGCAAGAAC ACACTGTATC TCCAAATGAA TAGCCTGCAA GCCGAGGACA CAGCCATATA

TTATTG
```

wps54

```
        10          20          30          40          50          60
ACACTCTAGA CCACCATGGC TGTCTTGGGG CTGCTCTTCT GCCTGGTGAC ATTCCCAAGC

        70          80          90         100         110         120
TGTGTCCTAT CCGCTGTCCA GCTGCTAGAG AGTGGTGGCG GTCTGGTGCA GCCAGGAGGA

       130
TCTCTGAGAC
```

wps57

```
        10          20          30          40          50          60
ACACTCTAGA AGTTAGGACT CACCTGAAGA GACAGTGACC AGAGTCCCTT GGCCCCAGTA

        70          80          90         100         110
AGCAAAACCG TCGTAATTAT AGTCCCCAGC TCTGGCACAA TAATATATGG CTGTGTCC
```

FIG. 11B

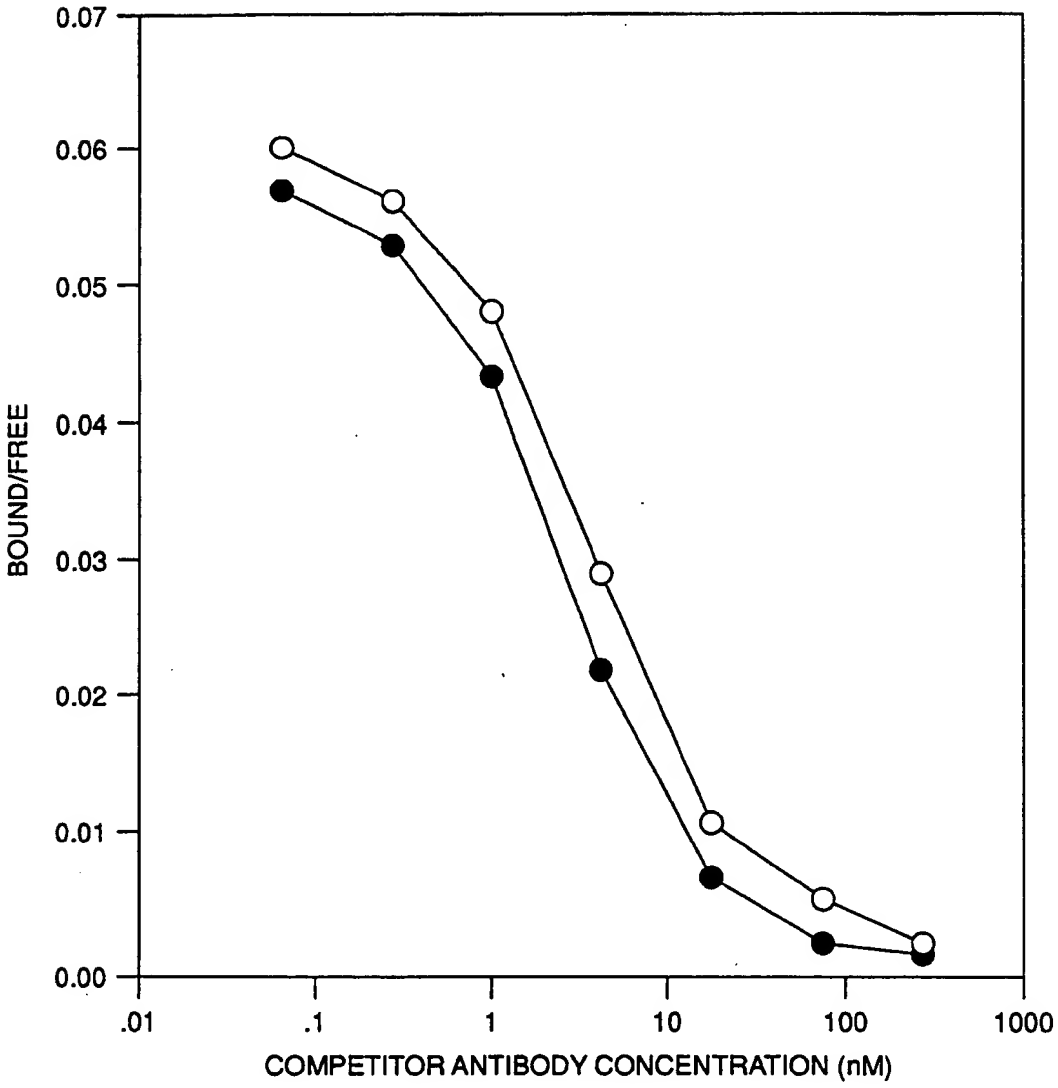
0.07
0.06
0.05
0.04
0.03
0.02
0.01
0.00
BOUND/FREE
.01
.1
1
10
100
1000
COMPETITOR ANTIBODY CONCENTRATION (nM)

FIG. 12

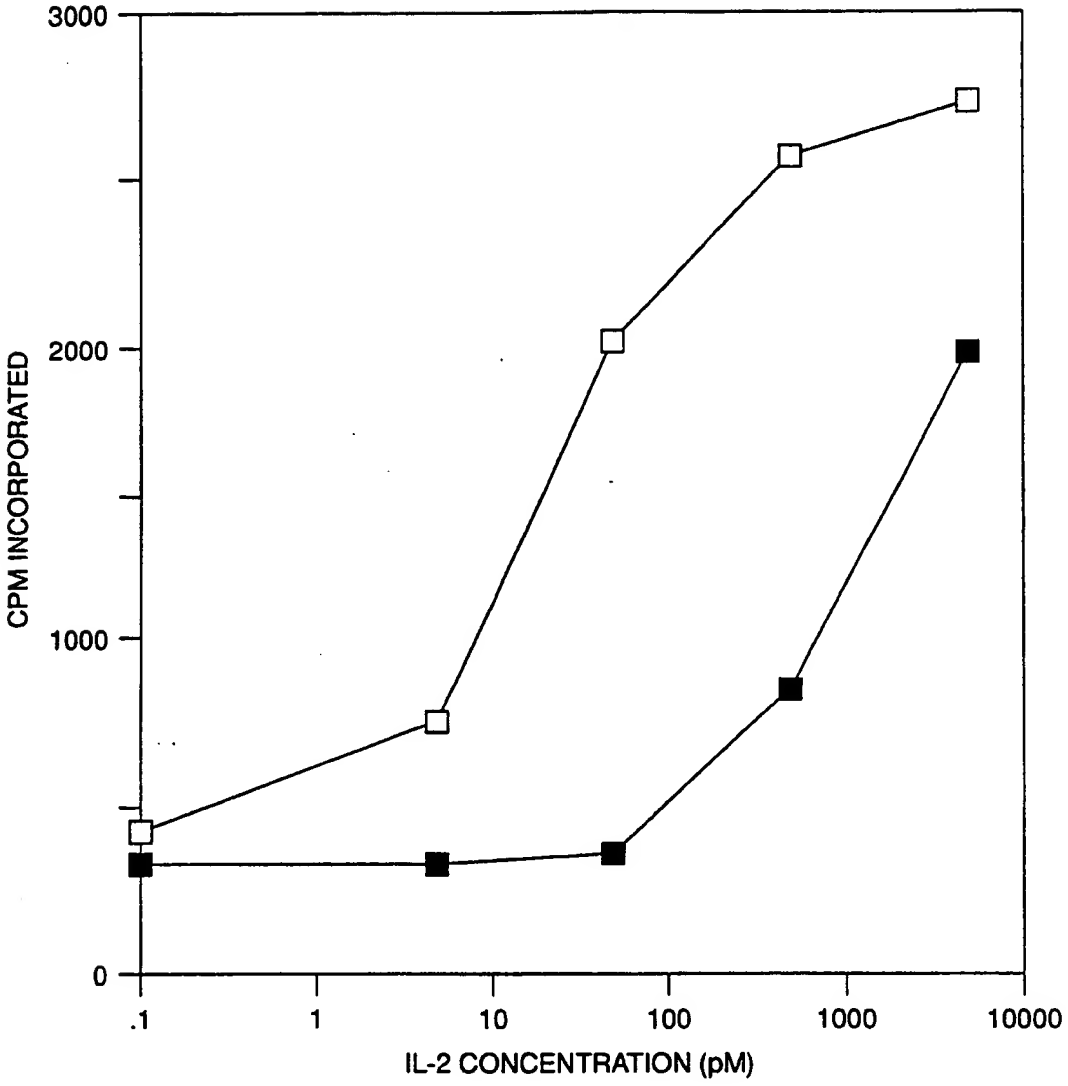
3000
2000
1000
0
CPM INCORPORATED
.1
1
10
100
1000
10000
IL-2 CONCENTRATION (pM)

FIG. 13

	1				5				10				15				20
1	E	M	I	L	V	E	S	G	G	G	L	V	K	P	G	A	S	L	K	L
1	E	V	Q	L	L	E	S	G	G	G	L	V	Q	P	G	G	S	L	R	L
					25				30				35				40
21	S	C	A	A	S	G	F	T	F	S	N	Y	G	L	S	W	V	R	Q	T
21	S	C	A	A	S	G	F	T	F	S	N	Y	G	L	S	W	V	R	Q	A
					45				50	52 a			55				
41	S	D	R	R	L	E	W	V	A	S	I	S	R	G	G	G	R	I	Y	S
41	P	G	K	G	L	E	W	V	A	S	I	S	R	G	G	G	R	I	Y	S
	60				65				70				75				
60	P	D	N	L	K	G	R	F	T	I	S	R	E	D	A	K	N	T	L	Y
60	P	D	N	L	K	G	R	F	T	I	S	R	N	D	S	K	N	T	L	Y
	80		82 a	b	c		85				90				95		
80	L	Q	M	S	S	L	K	S	E	D	T	A	L	Y	Y	C	L	R	E	G
80	L	Q	M	N	S	L	Q	A	E	D	T	A	L	Y	Y	C	L	R	E	G
			100 a	b	c	d	k			105				110			
97	I	Y	Y	A	D	Y	G	F	F	D	V	W	G	T	G	T	T	V	I	V
97	I	Y	Y	A	D	Y	G	F	F	D	V	W	G	Q	G	T	L	V	T	V
	113																
112	S	S															
112	S	S															

FIG. 14A

	1				5				10				15				20
1	D	I	V	L	T	Q	S	P	A	S	L	A	V	S	L	G	Q	R	A	T
1	E	I	V	M	T	Q	S	P	A	T	L	S	V	S	P	G	E	R	A	T
					25			27 a	b	c	d		30				35		
21	I	S	C	R	A	S	Q	S	V	S	T	S	T	Y	N	Y	M	H	W	Y
21	L	S	C	R	A	S	Q	S	V	S	T	S	T	Y	N	Y	M	H	W	Y
				40				45				50				55				
37	Q	Q	K	P	G	Q	P	P	K	L	L	I	K	Y	A	S	N	L	E	S
37	Q	Q	K	P	G	Q	S	P	R	L	L	I	K	Y	A	S	N	L	E	S
				60				65				70				75				
57	G	V	P	A	R	F	S	G	S	G	F	G	T	D	F	T	L	N	I	H
57	G	I	P	A	R	F	S	G	S	G	S	G	T	E	F	T	L	T	I	S
				80				85				90				95				
77	P	V	E	E	E	D	T	V	T	Y	Y	C	Q	H	S	W	E	I	P	Y
77	R	L	E	S	E	D	F	A	V	Y	Y	C	Q	H	S	W	E	I	P	Y
				100				105	107											
97	T	F	G	G	G	T	K	L	E	I	K									
97	T	F	G	Q	G	T	R	V	E	I	K									

FIG. 14B

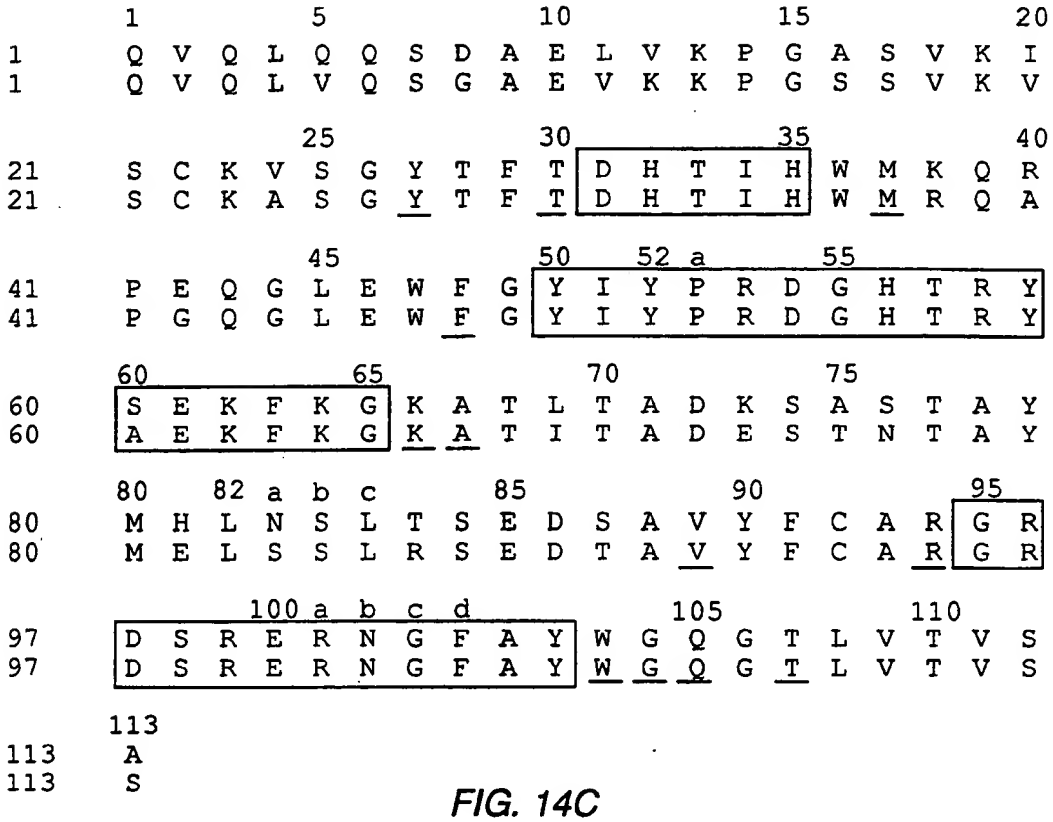

FIG. 14C

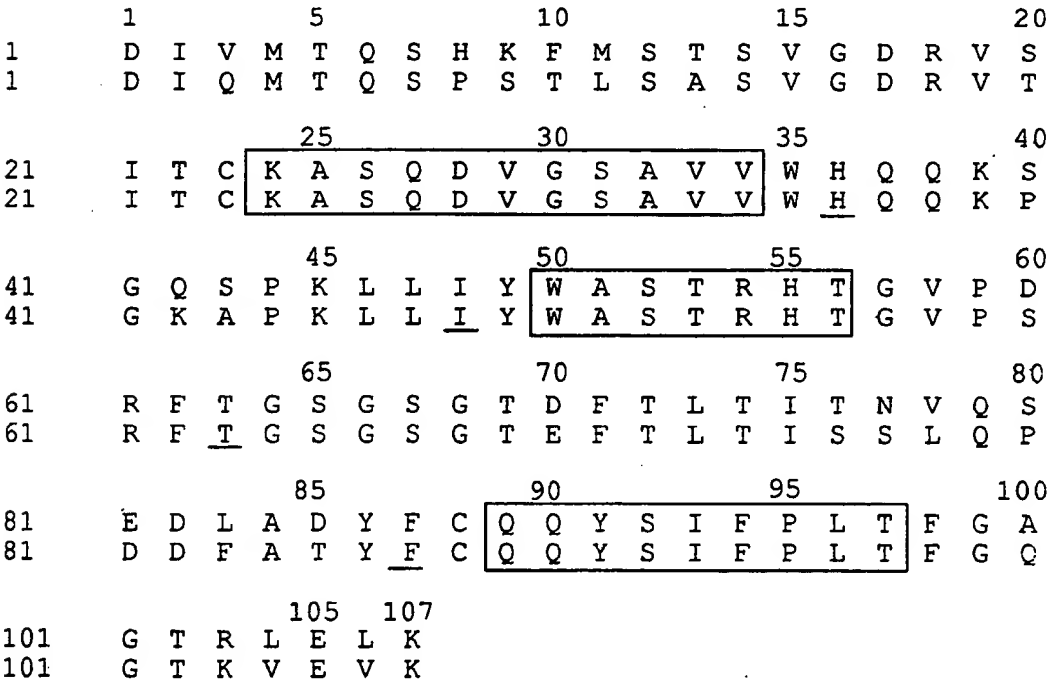

FIG. 14D

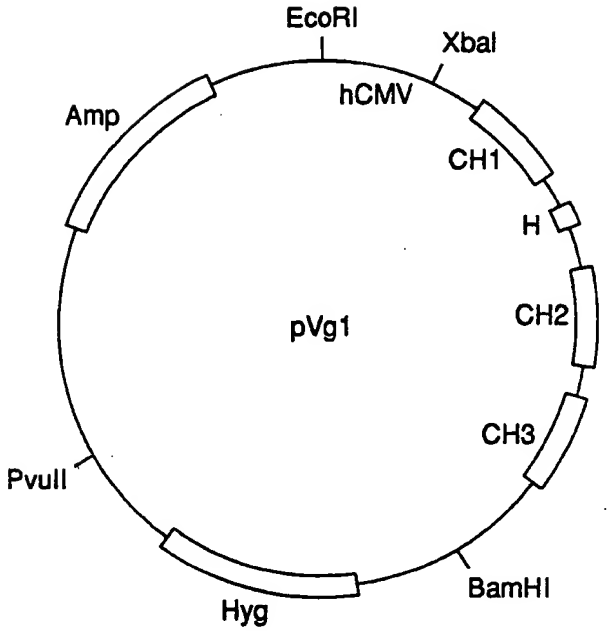

FIG. 15A

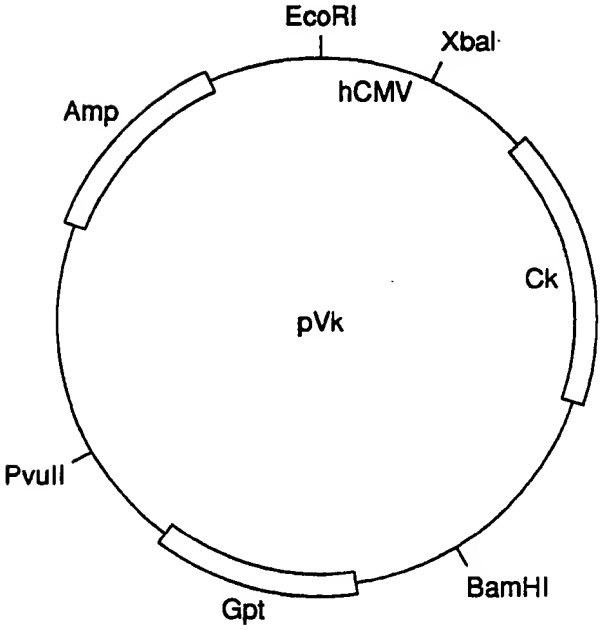

FIG. 15B

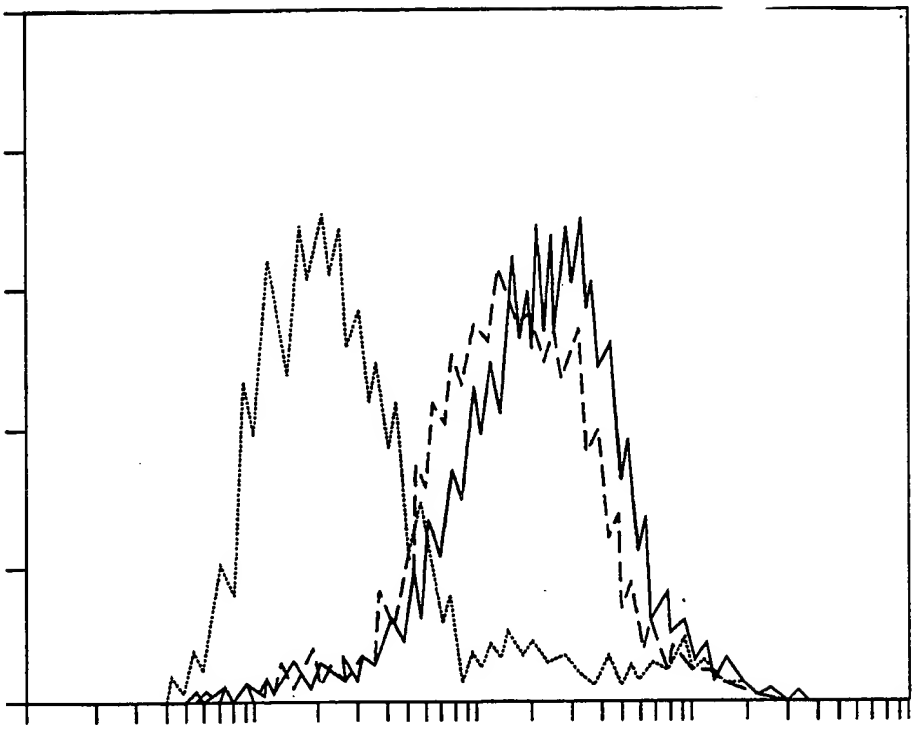

FIG. 16A

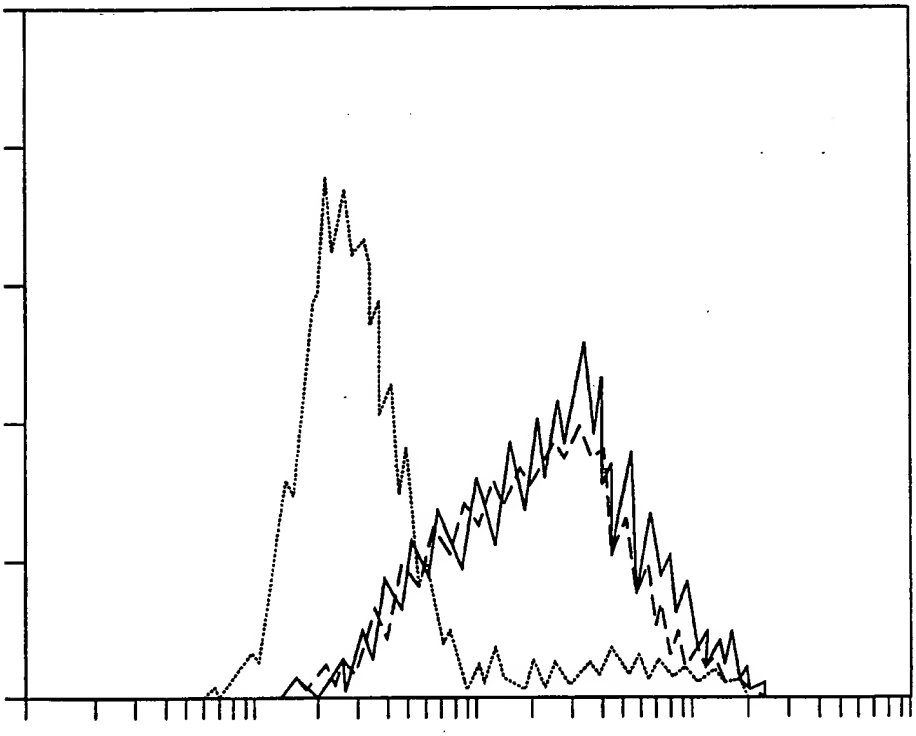

FIG. 16B

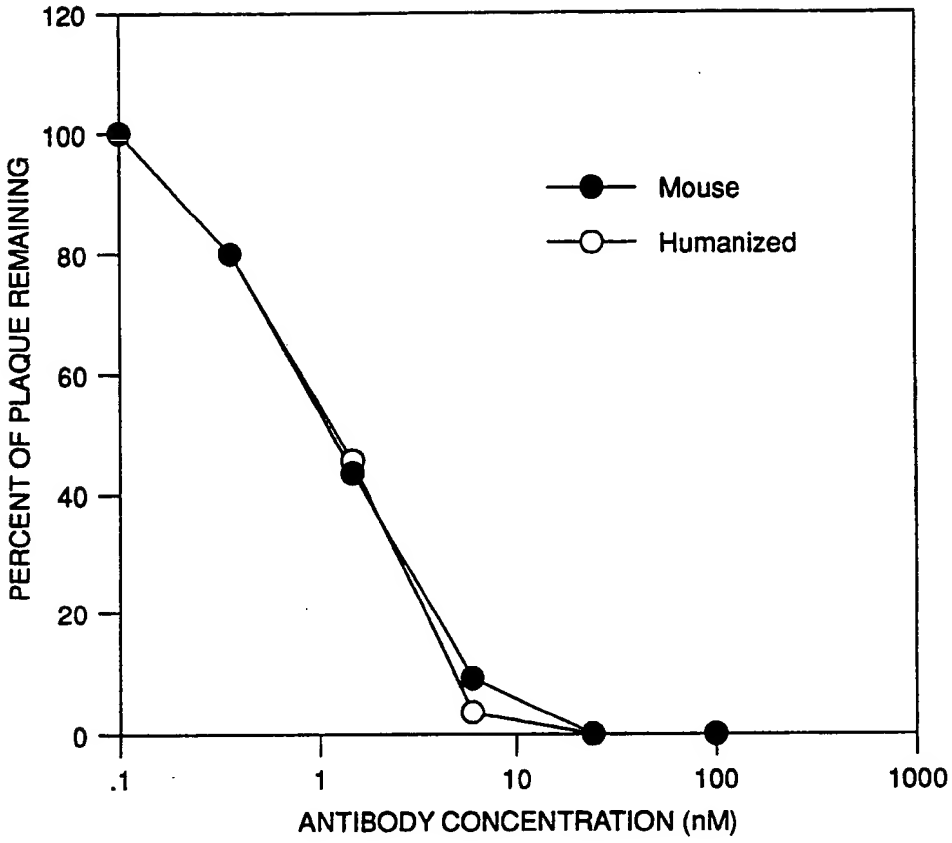
120
100
80
60
40
20
0
PERCENT OF PLAQUE REMAINING
Mouse
Humanized
.1
1
10
100
1000
ANTIBODY CONCENTRATION (nM)

FIG. 17A

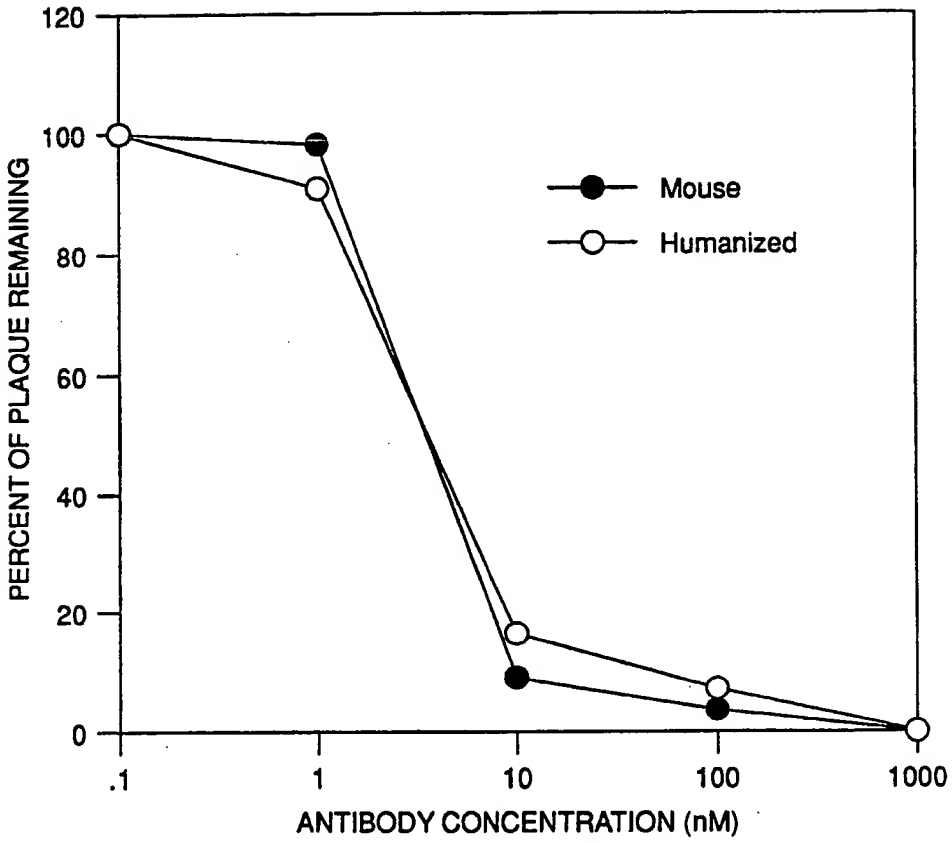
120
100
80
60
40
20
0
PERCENT OF PLAQUE REMAINING
Mouse
Humanized
.1
1
10
100
1000
ANTIBODY CONCENTRATION (nM)

FIG. 17B

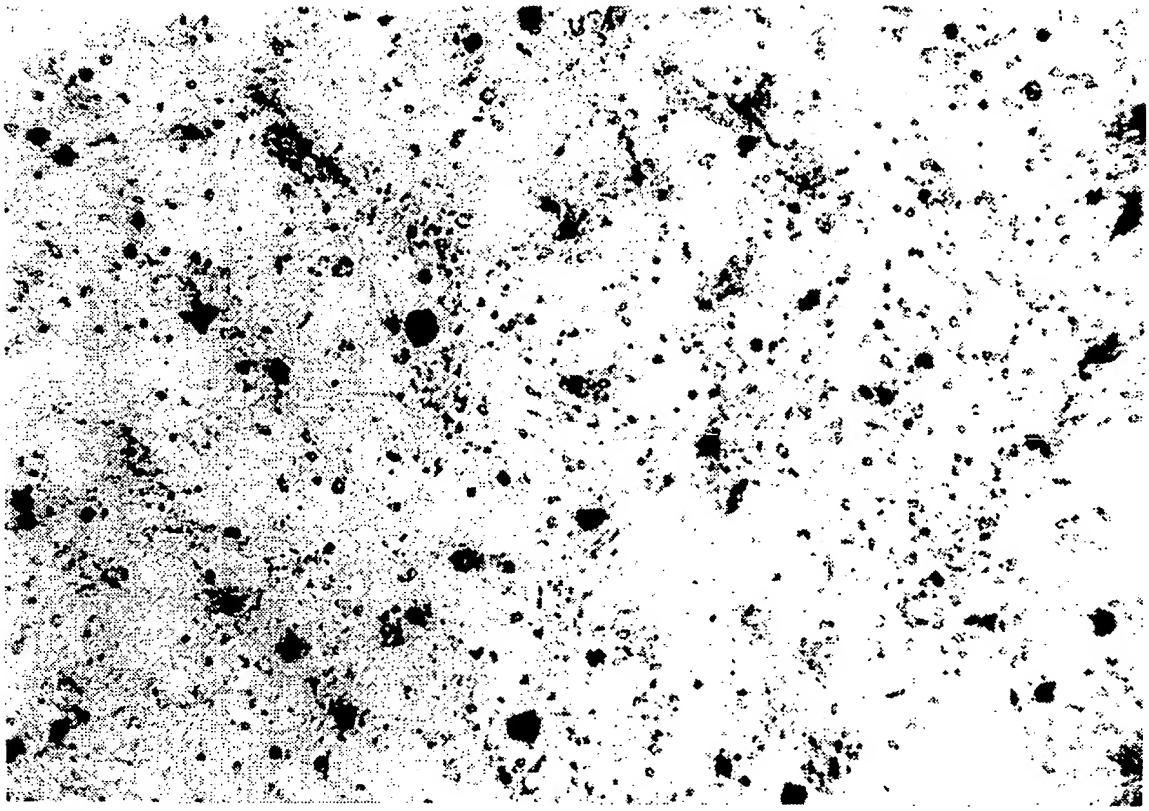

FIG. 18A

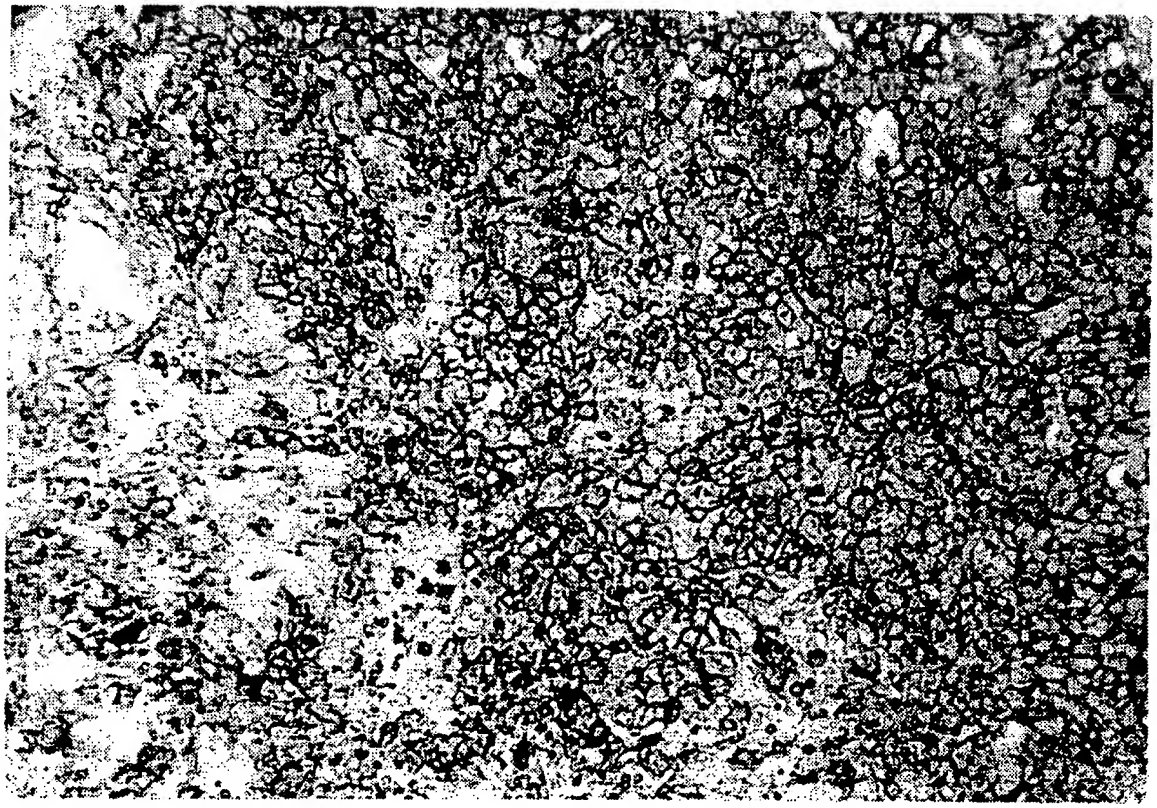

FIG. 18B

```
          .         .        30         .         .        60
ATGGAGAAAGACACACTCCTGCTATGGGTCCTGCTTCTCTGGGTTCCAGGTTCCACAGGT
 M  E  K  D  T  L  L  L  W  V  L  L  L  W  V  P  G  S  T  G

          .         .        90         .         .       120
GACATTGTGCTGACCCAATCTCCAGCTTCTTTGGCTGTGTCTCTAGGGCAGAGGGCCACC
 D  I  V  L  T  Q  S  P  A  S  L  A  V  S  L  G  Q  R  A  T

          .         .       150         .         .       180
ATCTCCTGCAGAGCCAGCGAAAGTGTTGATAATTATGGCATTAGTTTTATGAACTGGTTC
 I  S  C  R  A  S  E  S  V  D  N  Y  G  I  S  F  M  N  W  F

          .         .       210         .         .       240
CAACAGAAACCAGGACAGCCACCCAAACTCCTCATCTATGCTGCATCCAACCAAGGATCC
 Q  Q  K  P  G  Q  P  P  K  L  L  I  Y  A  A  S  N  Q  G  S

          .         .       270         .         .       300
GGGGTCCCTGCCAGGTTTAGTGGCAGTGGGTCTGGGACAGACTTCAGCCTCAACATCCAT
 G  V  P  A  R  F  S  G  S  G  S  G  T  D  F  S  L  N  I  H

          .         .       330         .         .       360
CCTATGGAGGAGGATGATACTGCAATGTATTTCTGTCAGCAAAGTAAGGAGGTTCCGTGG
 P  M  E  E  D  D  T  A  M  Y  F  C  Q  Q  S  K  E  V  P  W

          .         .       390
ACGTTCGGTGGAGGCACCAAGCTGGAAATCAAA
 T  F  G  G  G  T  K  L  E  I  K
```

FIG. 19A

```
          .         .        30         .         .        60
ATGGGATGGAGCTGGATCTTTCTCTTCCTCCTGTCAGGAACTGCAGGCGTCCACTCTGAG
 M  G  W  S  W  I  F  L  F  L  L  S  G  T  A  G  V  H  S  E

          .         .        90         .         .       120
GTCCAGCTTCAGCAGTCAGGACCTGAGCTGGTGAAACCTGGGGCCTCAGTGAAGATATCC
 V  Q  L  Q  Q  S  G  P  E  L  V  K  P  G  A  S  V  K  I  S

          .         .       150         .         .       180
TGCAAGGCTTCTGGATACACATTCACTGACTACAACATGCACTGGGTGAAGCAGAGCCAT
 C  K  A  S  G  Y  T  F  T  D  Y  N  M  H  W  V  K  Q  S  H

          .         .       210         .         .       240
GGAAAGAGCCTTGAGTGGATTGGATATATTTATCCTTACAATGGTGGTACTGGCTACAAC
 G  K  S  L  E  W  I  G  Y  I  Y  P  Y  N  G  G  T  G  Y  N

          .         .       270         .         .       300
CAGAAGTTCAAGAGCAAGGCCACATTGACTGTAGACAATTCCTCCAGCACAGCCTACATG
 Q  K  F  K  S  K  A  T  L  T  V  D  N  S  S  S  T  A  Y  M

          .         .       330         .         .       360
GACGTCCGCAGCCTGACATCTGAGGACTCTGCAGTCTATTACTGTGCAAGAGGGCGCCCC
 D  V  R  S  L  T  S  E  D  S  A  V  Y  Y  C  A  R  G  R  P

          .         .       390         .
GCTATGGACTACTGGGGTCAAGGAACCTCAGTCACCGTCTCCTCA
 A  M  D  Y  W  G  Q  G  T  S  V  T  V  S  S
```

FIG. 19B

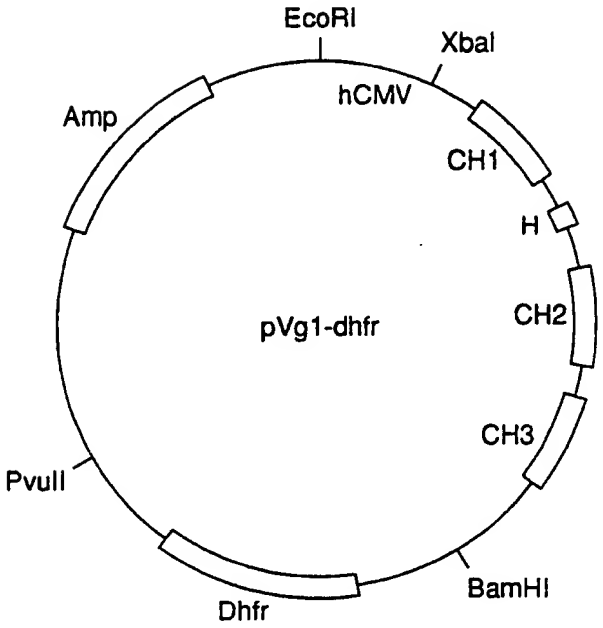

FIG. 20A

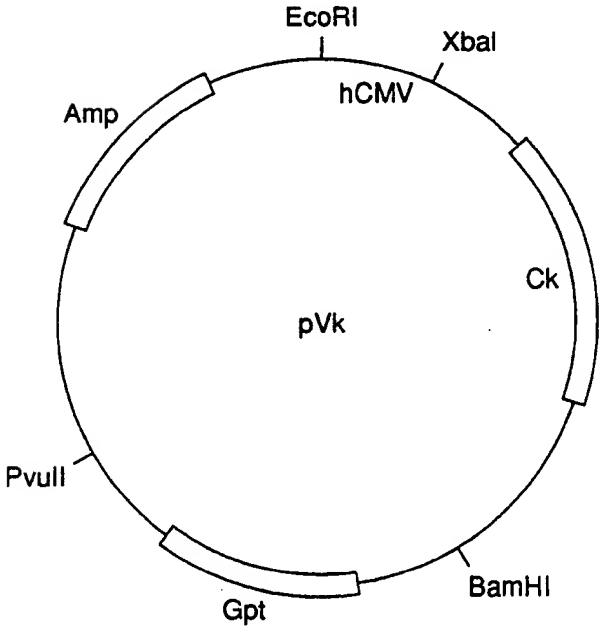

FIG. 20B

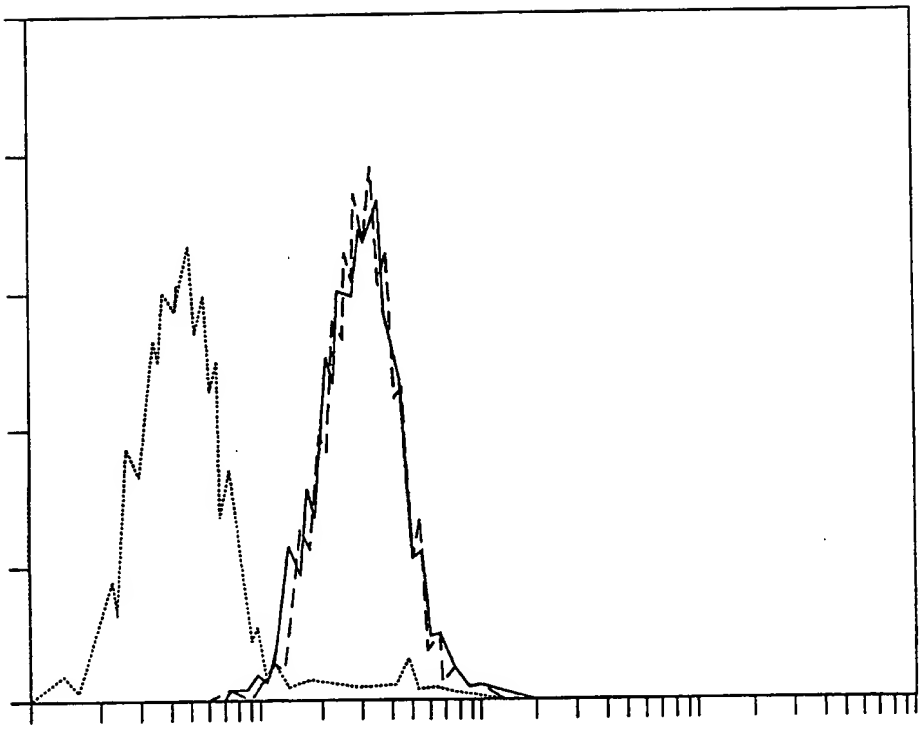

FIG. 21

1	D	I	Q	M	T	Q	S	P	S	T	L	S	A	S	V	G	D	R	V	T
1	D	I	Q	M	T	Q	S	P	S	S	L	S	A	S	V	G	D	R	V	T
21	I	T	C	R	A	S	Q	S		I	N				T	W	L	A	W	Y
21	I	T	C	R	A	S	E	S	V	D	N	Y	G	I	S	F	M	N	W	F
37	Q	Q	K	P	G	G	A	P	K	L	L	M	Y	K	A	S	S	L	E	S
41	Q	Q	K	P	G	G	A	P	K	L	L	I	Y	A	A	S	N	Q	G	S
57	G	V	P	S	R	F	I	G	S	G	S	G	T	E	F	T	L	T	I	S
61	G	V	P	S	R	F	S	G	S	G	S	G	T	D	F	T	L	T	I	S
77	S	L	Q	P	D	D	F	A	T	Y	Y	C	Q	Q	Y	N	S	D	S	K
81	S	L	Q	P	D	D	F	A	T	Y	Y	C	Q	Q	S	K	E	V	P	W
97	M	F	G	Q	G	T	K	V	E	V	K									
101	T	F	G	Q	G	T	K	V	E	I	K									

FIG. 22A

1	Q	V	Q	L	V	Q	S	G	A	E	V	K	K	P	G	S	S	V	K	V
1	Q	V	Q	L	V	Q	S	G	A	E	V	K	K	P	G	S	S	V	K	V
21	S	C	K	A	S	G	G	T	F	S	R	S	A	I	I	W	V	R	Q	A
21	S	C	K	A	S	G	Y	T	F	T	D	Y	N	M	H	W	V	R	Q	A
41	P	G	Q	G	L	E	W	M	G	G	I	V	P	M	F	G	P	P	N	Y
41	P	G	Q	G	L	E	W	I	G	Y	I	Y	P	Y	N	G	G	T	G	Y
61	A	Q	K	F	Q	G	R	V	T	I	T	A	D	E	S	T	N	T	A	Y
61	N	Q	K	F	K	S	K	A	T	I	T	A	D	E	S	T	N	T	A	Y
81	M	E	L	S	S	L	R	S	E	D	T	A	F	Y	F	C	A	G	G	Y
81	M	E	L	S	S	L	R	S	E	D	T	A	V	Y	Y	C	A	R	G	
101	G	I	Y	S	P	E	E	Y	N	G	G	L	V	T	V	S	S			
100	R	P	A	M	D	Y	W	G	Q	G	T	L	V	T	V	S	S			

FIG. 22B

ma1

```
        10          20          30          40          50          60
TATATCTAGA CCACCATGGG ATGGAGCTGG ATCTTTCTCT TCCTCCTGTC AGGAACTGCT

        70          80          90         100         110         120
GGCGTCCACT CTCAGGTTCA GCTGGTGCAG TCTGGAGCTG AGGTGAAGAA GCCTGGGAGC

       130
TCAGTGAAGG TT
```

ma2

```
        10          20          30          40          50          60
AGCCGGTACC ACCATTGTAA GGATAAATAT ATCCAATCCA TTCCAGGCCT TGGCCAGGAG

        70          80          90         100         110         120
CCTGCCTCAC CCAGTGCATG TTGTAGTCAG TGAAGGTGTA GCCAGAAGCT TTGCAGGAAA

       130
CCTTCACTGA GCT
```

ma3

```
        10          20          30          40          50          60
TGGTGGTACC GGCTACAACC AGAAGTTCAA GAGCAAGGCC ACAATTACAG CAGACGAGAG

        70          80          90         100         110
TACTAACACA GCCTACATGG AACTCTCCAG CCTGAGGTCT GAGGACACTG CA
```

ma4

```
        10          20          30          40          50          60
TATATCTAGA GGCCATTCTT ACCTGAAGAG ACAGTGACCA GAGTCCCTTG GCCCCAGTAG

        70          80          90         100         110
TCCATAGCGG GGCGCCCTCT TGCGCAGTAA TAGACTGCAG TGTCCTCAGA C
```

FIG. 23A

ma5

```
        10          20          30          40          50          60
TATATCTAGA CCACCATGGA GAAAGACACA CTCCTGCTAT GGGTCCTGCT TCTCTGGGTT

        70          80          90         100         110         120
CCAGGTTCCA CAGGTGACAT TCAGATGACC CAGTCTCCGA GCTCTCTGTC CGCATCAGTA

GG
```

ma6

```
        10          20          30          40          50          60
TCAGAAGCTT AGGAGCCTTC CCGGGTTTCT GTTGGAACCA GTTCATAAAG CTAATGCCAT

        70          80          90         100         110         120
AATTGTCGAC ACTTTCGCTG GCTCTGCATG TGATGGTGAC CCTGTCTCCT ACTGATGCGG

AC
```

ma7

```
        10          20          30          40          50          60
TCCTAAGCTT CTGATTTACG CTGCATCCAA CCAAGGCTCC GGGGTACCCT CTCGCTTCTC

        70          80          90         100         110
AGGCAGTGGA TCTGGGACAG ACTTCACTCT CACCATTTCA TCTCTGCAGC CTGATGACT
```

ma8

```
        10          20          30          40          50          60
TATATCTAGA CTTTGGATTC TACTTACGTT TGATCTCCAC CTTGGTCCCT TGACCGAACG

        70          80          90         100         110
TCCACGGAAC CTCCTTACTT TGCTGACAGT AATAGGTTGC GAAGTCATCA GGCTGCAG
```

FIG. 23B

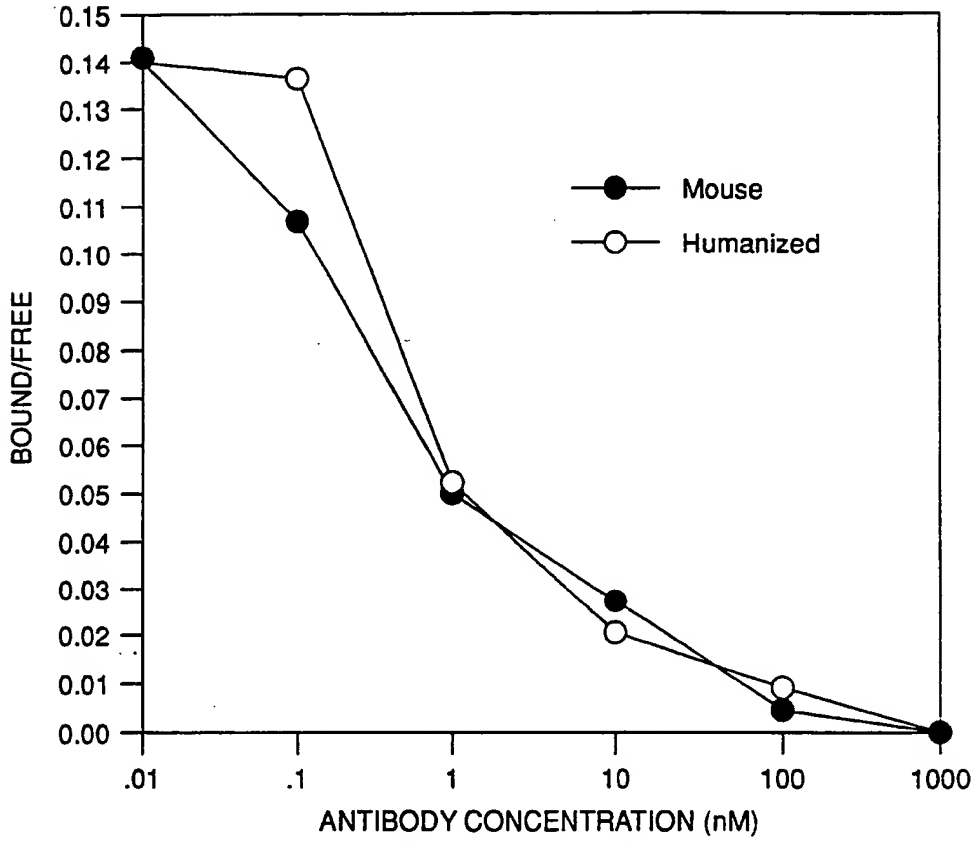
0.15
0.14
0.13
0.12
0.11
0.10
0.09
0.08
0.07
0.06
0.05
0.04
0.03
0.02
0.01
0.00
BOUND/FREE
Mouse
Humanized
.01
.1
1
10
100
1000
ANTIBODY CONCENTRATION (nM)

FIG. 24

```
                    .         .        30         .         .        60
ATGGTTTTCACACCTCAGATACTTGGACTTATGCTTTTTTGGATTTCAGCCTCCAGAGGT
 M  V  F  T  P  Q  I  L  G  L  M  L  F  W  I  S  A  S  R  G

                    .         .        90         .         .       120
GATATTGTGCTAACTCAGTCTCCAGCCACCCTGTCTGTGACTCCGGGAGATAGCGTCAGT
 D  I  V  L  T  Q  S  P  A  T  L  S  V  T  P  G  D  S  V  S

                    .         .       150         .         .       180
CTTTCCTGCAGGGCCAGCCAAAGTATTAGCAACAACCTACACTGGTATCAACAAAAATCA
 L  S  C  R  A  S  Q  S  I  S  N  N  L  H  W  Y  Q  Q  K  S

                    .         .       210         .         .       240
CATGAGTCTCCAAGGCTTCTCATCAAGTATGCTTCCCAGTCCATCTCTGGGATCCCCTCC
 H  E  S  P  R  L  L  I  K  Y  A  S  Q  S  I  S  G  I  P  S

                    .         .       270         .         .       300
AGGTTCAGTGGCAGTGGATCAGGGACAGATTTCACTCTCAGTGTCAACGGTGTGGAGACT
 R  F  S  G  S  G  S  G  T  D  F  T  L  S  V  N  G  V  E  T

                    .         .       330         .         .       360
GAAGATTTTGGAATGTATTTCTGTCAACACACTAACAGTTGGCCTCATACGTTCGGAGGG
 E  D  F  G  M  Y  F  C  Q  Q  S  N  S  W  P  H  T  F  G  G

                    .         .
GGGACCAAGCTGGAAATAAAA
 G  T  K  L  E  I  K
```

FIG. 25A

```
                    .         .        30         .         .        60
ATGGGATGGAGCTGGATCTTTCTCTTCCTCCTGTCAGGAACTGCAGGTGTCCACTCTGAG
 M  G  W  S  W  I  F  L  F  L  L  S  G  T  A  G  V  H  S  E

                    .         .        90         .         .       120
GTCCAGCTGCAACAGTCTGGACCTGAGCTGGTGAAGCCTGGAGCTTCAATGAAGATATCC
 V  Q  L  Q  Q  S  G  P  E  L  V  K  P  G  A  S  M  K  I  S

                    .         .       150         .         .       180
TGCAAGGCTTCTGTTTACTCATTCACTGGCTACACCATGAACTGGGTGAAGCAGAGCCAT
 C  K  A  S  V  Y  S  F  T  G  Y  T  M  N  W  V  K  Q  S  H

                    .         .       210         .         .       240
GGACAGAACCTTGAGTGGATTGGACTTATTAATCCTTACAATGGTGGTACTAGCTACAAC
 G  Q  N  L  E  W  I  G  L  I  N  P  Y  N  G  G  T  S  Y  N

                    .         .       270         .         .       300
CAGAAGTTCAAGGGGAAGGCCACATTAACTGTAGACAAGTCATCCAACACAGCCTACATG
 Q  K  F  K  G  K  A  T  L  T  V  D  K  S  S  N  T  A  Y  M

                    .         .       330         .         .       360
GAGCTCCTCAGTCTGACATCTGCGGACTCTGCAGTCTATTACTGTACAAGACGGGGGTTT
 E  L  L  S  L  T  S  A  D  S  A  V  Y  Y  C  T  R  R  G  F

                    .         .       390         .         .
CGAGACTATTCTATGGACTACTGGGGTCAAGGAACCTCAGTCACCGTCTCCTCA
 R  D  Y  S  M  D  Y  W  G  Q  G  T  S  V  T  V  S  S
```

FIG. 25B

SUBSTITUTE SHEET

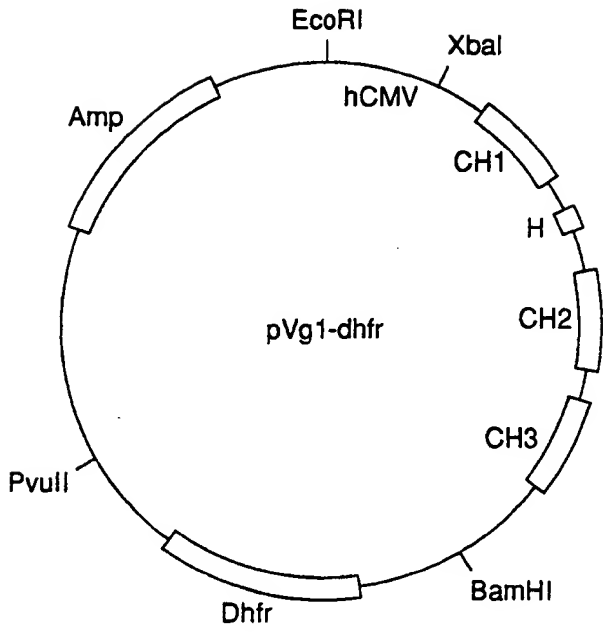

FIG. 26A

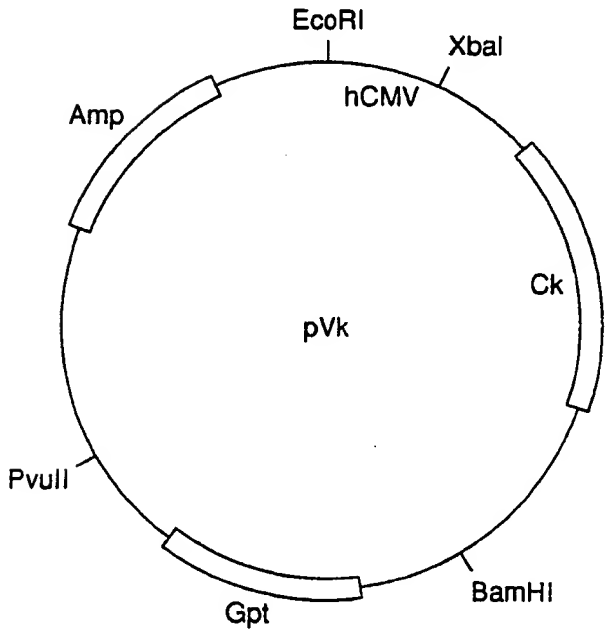

FIG. 26B

1	E	I	V	L	T	Q	S	P	G	T	L	S	L	S	P	G	E	R	A	T
1	E	I	V	L	T	Q	S	P	G	T	L	S	L	S	P	G	E	R	A	T
21	L	S	C	R	A	S	Q	S	V	S	S	G	Y	L	G	W	Y	Q	Q	K
21	L	S	C	R	A	S	Q	S		I	S	N	N	L	H	W	Y	Q	Q	K
41	P	G	Q	A	P	R	L	L	I	Y	G	A	S	S	R	A	T	G	I	P
40	P	G	Q	A	P	R	L	L	I	K	Y	A	S	Q	S	I	S	G	I	P
61	D	R	F	S	G	S	G	S	G	T	D	F	T	L	T	I	S	R	L	E
60	D	R	F	S	G	S	G	S	G	T	D	F	T	L	T	I	S	R	L	E
81	P	E	D	F	A	V	Y	Y	C	Q	Q	Y	G	S	L	G	R	T	F	G
80	P	E	D	F	A	V	Y	Y	C	Q	Q	S	N	S	W	P	H	T	F	G
101	Q	G	T	K	V	E	I	K												
100	Q	G	T	K	V	E	I	K												

FIG. 27A

1	Q	V	Q	L	M	Q	S	G	A	E	V	K	K	P	G	S	S	V	R	V
1	Q	V	Q	L	V	Q	S	G	A	E	V	K	K	P	G	S	S	V	R	V
21	S	C	K	T	S	G	G	T	F	V	D	Y	K	G	L	W	V	R	Q	A
21	S	C	K	A	S	G	Y	S	F	T	G	Y	T	M	N	W	V	R	Q	A
41	P	G	K	G	L	E	W	V	G	Q	I	P	L	R	F	N	G	E	V	K
41	P	G	K	G	L	E	W	V	G	L	I	N	P	Y	N	G	G	T	S	Y
61	N	P	G	S	V	V	R	V	S	V	S	L	K	P	S	F	N	Q	A	H
61	N	Q	K	F	K	G	R	V	T	V	S	L	K	P	S	F	N	Q	A	Y
81	M	E	L	S	S	L	F	S	E	D	T	A	V	Y	Y	C	A	R	E	Y
81	M	E	L	S	S	L	F	S	E	D	T	A	V	Y	Y	C	T	R	R	
101	G	F	D	T	S	D	Y	Y	Y	Y	Y	W	G	Q	G	T	L	V	T	V
100	G	F			R	D	Y	S	M	D	Y	W	G	Q	G	T	L	V	T	V
121	S	S																		
118	S	S																		

FIG. 27B

jb16

```
        10          20          30          40          50          60
TAGATCTAGA CCACCATGGT TTTCACACCT CAGATACTAG GACTCATGCT CTTCTGGATT

        70          80          90          100         110         120
TCAGCCTCCA GAGGTGAAAT TGTGCTAACT CAGTCTCCAG GCACCCTAAG CTTATCACCG

GGAGAAAGG
```

jb17

```
        10          20          30          40          50          60
TAGACAGAAT TCACGCGTAC TTGATAAGTA GACGTGGAGC TTGTCCAGGT TTTTGTTGGT

        70          80          90          100         110         120
ACCAGTGTAG GTTGTTGCTA ATACTTTGGC TGGCCCTGCA GGAAAGTGTA GCCCTTTCTC

CCGGTGAT
```

jb18

```
        10          20          30          40          50          60
AAGAGAATTC ACGCGTCCCA GTCCATCTCT GGAATACCCG ATAGGTTCAG TGGCAGTGGA

        70          80          90          100         110
TCAGGGACAG ATTTCACTCT CACAATAAGT AGGCTCGAGC CGGAAGATTT TGC
```

jb19

```
        10          20          30          40          50          60
TAGATCTAGA GTTGAGAAGA CTACTTACGT TTTATTTCTA CCTTGGTCCC TTGTCCGAAC

        70          80          90          100         110
GTATGAGGCC AACTGTTACT CTGTTGACAA TAATACACAG CAAAATCTTC CGGCTC
```

FIG. 28A

jb20

```
        10          20          30          40          50          60
TATATCTAGA CCACCATGGG ATGGAGCTGG ATCTTTCTCT TCCTCCTGTC AGGAACTGCA

        70          80          90         100         110         120
GGTGTCCACT CTCAAGTCCA ACTGGTACAG TCTGGAGCTG AGGTTAAAAA GCCTGGAAGT

       130
TCAGTAAGAG TTTC
```

jb21

```
        10          20          30          40          50          60
TATATAGGTA CCACCATTGT AAGGATTAAT AAGTCCAACC CACTCAAGTC CTTTTCCAGG

        70          80          90         100         110         120
TGCCTGTCTC ACCCAGTTCA TGGTATACCC AGTGAATGAG TATCCGGAAG CTTTGCAGGA

       130
AACTCTTACT GAAC
```

jb22

```
        10          20          30          40          50          60
TATATAGGTA CCAGCTACAA  CCAGAAGTTC AAGGGCACAG TTACAGTTC TTTGAAGCCT

        70          80          90         100         110
TCATTTAACC AGGCCTACAT GGAGCTCAGT AGTCTGTTTT CTGAAGACAC TGCAGT
```

jb23

```
        10          20          30          40          50          60
TATATCTAGA GGCCATTCTT ACCTGAGGAG ACGGTGACTA AGGTTCCTTG ACCCCAGTAG

        70          80          90         100         110
TCCATAGAAT AGTCTCGAAA CCCCCGTCTT CTACAGTAAT AGACTGCAGT GTCTTC
```

FIG. 28B

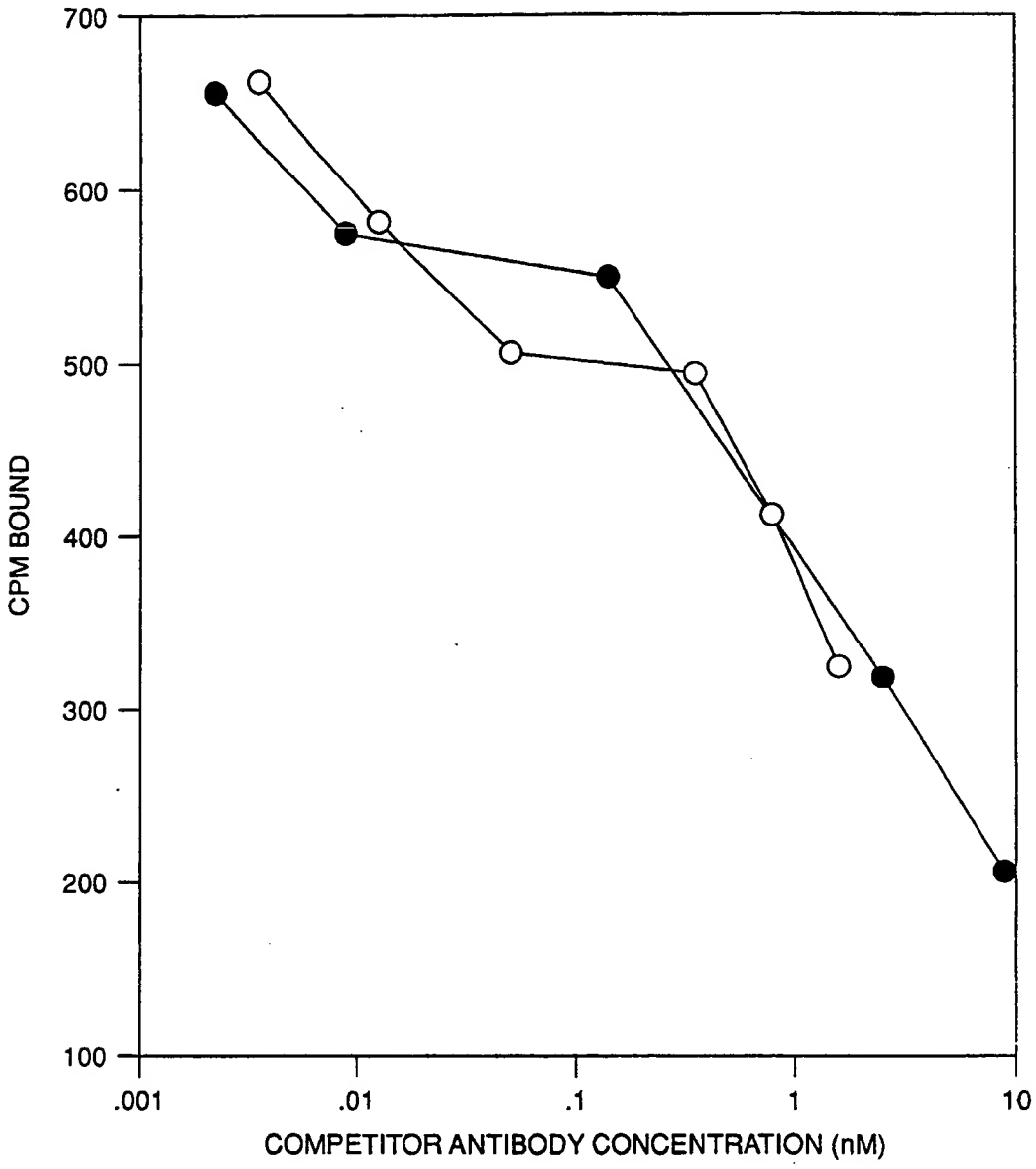
700
600
500
400
300
200
100
CPM BOUND
.001
.01
.1
1
10
COMPETITOR ANTIBODY CONCENTRATION (nM)

FIG. 29

```
          .         .        30         .         .        60
ATGACATCACTGTTCTCTCTACAGTTACCGAGCACACAGGACCTCGCCATGGGATGGAGC
 M  T  S  L  F  S  L  Q  L  P  S  T  Q  D  L  A  M  G  W  S

          .         .        90         .         .       120
TGTATCATCCTCTTCTTGGTAGCAACAGCTACAGGTGTCCTCTCCCAGGTCCAACTGCAG
 C  I  I  L  F  L  V  A  T  A  T  G  V  L  S  Q  V  Q  L  Q

          .         .       150         .         .       180
CAGCCTGGGGCTGACCTTGTGATGCCTGGGGCTCCAGTGAAGCTGTCCTGCTTGGCTTCT
 Q  P  G  A  D  L  V  M  P  G  A  P  V  K  L  S  C  L  A  S

          .         .       210         .         .       240
GGCTACATCTTCACCAGCTCCTGGATAAACTGGGTGAAGCAGAGGCCTGGACGAGGCCTC
 G  Y  I  F  T  S  S  W  I  N  W  V  K  Q  R  P  G  R  G  L

          .         .       270         .         .       300
GAGTGGATTGGAAGGATTGATCCTTCCGATGGTGAAGTTCACTACAATCAAGATTTCAAG
 E  W  I  G  R  I  D  P  S  D  G  E  V  H  Y  N  Q  D  F  K

          .         .       330         .         .       360
GACAAGGCCACACTGACTGTAGACAAATCCTCCAGCACAGCCTACATCCAACTCAACAGC
 D  K  A  T  L  T  V  D  K  S  S  S  T  A  Y  I  Q  L  N  S

          .         .       390         .         .       420
CTGACATCTGAGGACTCTGCGGTCTATTACTGTGCTAGAGGATTTCTGCCCTGGTTTGCT
 L  T  S  E  D  S  A  V  Y  Y  C  A  R  G  F  L  P  W  F  A

          .         .       450
GACTGGGGCCAAGGGACTCTGGTCACTGTCTCTGCA
 D  W  G  Q  G  T  L  V  T  V  S  A
```

FIG. 30A

```
          .         .        30         .         .        60
ATGCATCAGACCAGCATGGGCATCAAGATGGAATCACAGACTCTGGTCTTCATATCCATA
 M  H  Q  T  S  M  G  I  K  M  E  S  Q  T  L  V  F  I  S  I

          .         .        90         .         .       120
CTGCTCTGGTTATATGGTGCTCATGGGAACATTGTTATGACCCAATCTCCCAAATCCATG
 L  L  W  L  Y  G  A  D  G  N  I  V  M  T  Q  S  P  K  S  M

          .         .       150         .         .       180
TACGTGTCAATAGGAGAGAGGGTCACCTTGAGCTGCAAGGCCAGTGAAAATGTGGATACT
 Y  V  S  I  G  E  R  V  T  L  S  C  K  A  S  E  N  V  D  T

          .         .       210         .         .       240
TATGTATCCTGGTATCAACAGAAACCAGAGCAGTCTCCTAAACTGCTGATATATGGGGCA
 Y  V  S  W  Y  Q  Q  K  P  E  Q  S  P  K  L  L  I  Y  G  A

          .         .       270         .         .       300
TCCAACCGGTACACTGGGGTCCACGATCGCTTCACGGGCAGTGGATCTGCAACAGATTTC
 S  N  R  Y  T  G  V  H  D  R  F  T  G  S  G  S  A  T  D  F

          .         .       330         .         .       360
ACTCTGACCATCAGCAGTGTGCAGGCTGAAGACCTTGCAGATTATCACTGTGGACAGAGT
 T  L  T  I  S  S  V  Q  A  E  D  L  A  D  Y  H  C  G  Q  S

          .         .       390         .
TACAACTATCCATTCACGTTCGGCTCGGGGACAAAGTTGGAAATAAAG
 Y  N  Y  P  F  T  F  G  S  G  T  K  L  E  I  K
```

FIG. 30B

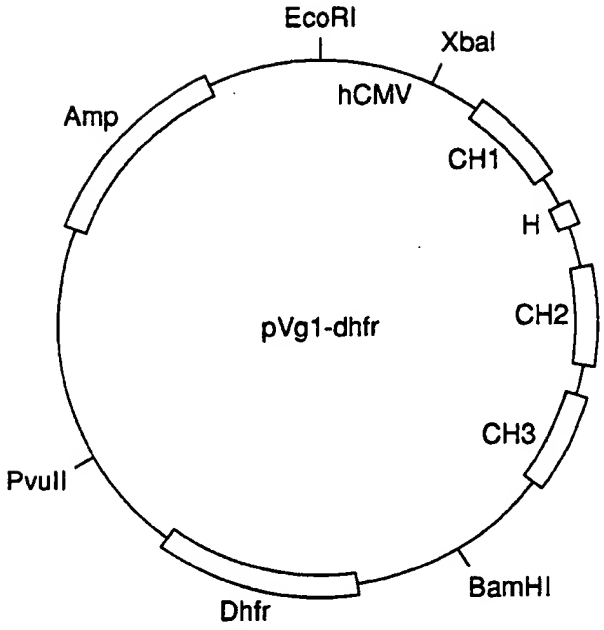

FIG. 31A

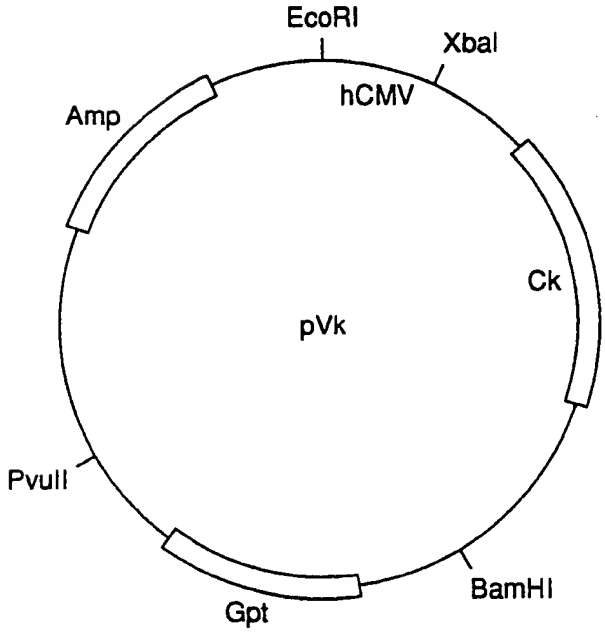

FIG. 31B

1	D	I	Q	M	T	Q	S	P	S	T	L	S	A	S	V	G	D	R	V	T
1	D	I	Q	M	T	Q	S	P	S	T	L	S	A	S	V	G	D	R	V	T
21	I	T	C	R	A	S	Q	S	I	N	T	W	L	A	W	Y	Q	Q	K	P
21	I	T	C	K	A	S	E	N	V	D	T	Y	V	S	W	Y	Q	Q	K	P
41	G	K	A	P	K	L	L	M	Y	K	A	S	S	L	E	S	G	V	P	S
41	G	K	A	P	K	L	L	I	Y	G	A	S	N	R	Y	T	G	V	P	S
61	R	F	I	G	S	G	S	G	T	E	F	T	L	T	I	S	S	L	Q	P
61	R	F	S	G	S	G	S	G	T	D	F	T	L	T	I	S	S	L	Q	P
81	D	D	F	A	T	Y	Y	C		Q	Q	Y	N	S	D	S	K	M	F	G
81	D	D	F	A	T	Y	Y	C	G	Q	S	Y	N		Y	P	F	T	F	G
100	Q	G	T	K	V	E	V	K												
100	Q	G	T	K	V	E	V	K												

FIG. 32A

1	Q	V	Q	L	V	Q	S	G	A	E	V	K	K	P	G	S	S	V	K	V
1	Q	V	Q	L	V	Q	S	G	A	E	V	K	K	P	G	S	S	V	K	V
21	S	C	K	A	S	G	G	T	F	S	R	S	A	I	I	W	V	R	Q	A
21	S	C	K	A	S	G	Y	I	F	T	S	S	W	I	N	W	V	R	Q	A
41	P	G	Q	G	L	E	W	M	G	G	I	V	P	M	F	G	P	P	N	Y
41	P	G	Q	G	L	E	W	M	G	R	I	D	P	S	D	G	E	V	H	Y
61	A	Q	K	F	Q	G	R	V	T	I	T	A	D	E	S	T	N	T	A	Y
61	N	Q	D	F	K	D	R	V	T	I	T	A	D	E	S	T	N	T	A	Y
81	M	E	L	S	S	L	R	S	E	D	T	A	F	Y	F	C	A	G	G	Y
81	M	E	L	S	S	L	R	S	E	D	T	A	V	Y	Y	C	A	R	G	F
101	G	I	Y	S	P	E	E	Y	N	G	G	L	V	T	V	S	S			
101	L	P	W	F	A	D	W	G	Q	G	T	L	V	T	V	S	S			

FIG. 32B

rh10

```
        10          20          30          40          50          60
TTTTTTCTAG ACCACCATGG AGACCGATAC CCTCCTGCTA TGGGTCCTCC TGCTATGGGT

        70          80          90         100         110
CCCAGGATCA ACCGGAGATA TTCAGATGAC CCAGTCTCCG TCGACCCTCT CTGCT
```

rh11

```
        10          20          30          40          50          60
TTTTAAGCTT GGGAGCTTTG CCTGGCTTCT GCTGATACCA GGATACATAA GTATCCACAT

        70          80          90         100         110         120
TTTCACTGGC CTTGCAGGTT ATGGTGACCC TATCCCCGAC GCTAGCAGAG AGGTTCCACG
```

rh12

```
        10          20          30          40          50          60
TTTTAAGCTT CTAATTTATG GGGCATCCAA CCGGTACACT GGGGTACCTT CACGCTTCAG

        70          80          90         100         110
TGGCAGTGGA TCTGGGACCG ATTTCACCCT CACAATCAGC TCTCTGCAGC CAGATGAT
```

rh13

```
        10          20          30          40          50          60
TTTTTTCTAG AGCAAAAGTC TACTTACGTT TGACCTCCAC CTTGGTCCCC TGACCGAACG

        70          80          90         100         110         120
TGAATGGATA GTTGTAACTC TGTCCGCAGT AATAAGTGGC GAAATCATCT GGCTCCAGAG
```

FIG. 33A

rh20

```
        10          20          30          40          50          60
TTTTTCTAGA CCACCATGGG ATGGAGCTGG ATCTTTCTCT TCCTCCTGTC AGGTACCGCG

        70          80          90         100         110
GGCGTGCACT CTCAGGTCCA GCTTGTCCAG TCTGGGGCTG AAGTCAAGAA ACCT
```

rh21

```
        10          20          30          40          50          60
TTTTGAATTC TCGAGACCCT GTCCAGGGGC CTGCCTTACC CAGTTTATCC AGGAGCTAGT

        70          80          90         100         110         120
AAAGATGTAG CCAGAAGCTT TGCAGGAGAC CTTCACGGAG CTCCCAGGTT TCTTGACTTC

A
```

rh22

```
        10          20          30          40          50          60
TTTTGAATTC TCGAGTGGAT GGGAAGGATT GATCCTTCCG ATGGTGAAGT TCACTACAAT

        70          80          90         100         110         120
CAAGATTTCA AGGACCGTGT TACAATTACA GCAGACGAAT CCACCAATAC AGCCTACATG

       130
GAACTGAGCA GCCTGAG
```

rh23

```
        10          20          30          40          50          60
TTTTTCTAGA GGTTTTAAGG ACTCACCTGA GGAGACTGTG ACCAGGGTTC CTTGGCCCCA

        70          80          90         100         110         120
GTCAGCAAAC CAGGGCAGAA ATCCTCTTGC ACAGTAATAG ACTGCAGTGT CCTCTGATCT

       130
CAGGCTGCTC AGTT
```

FIG. 33B

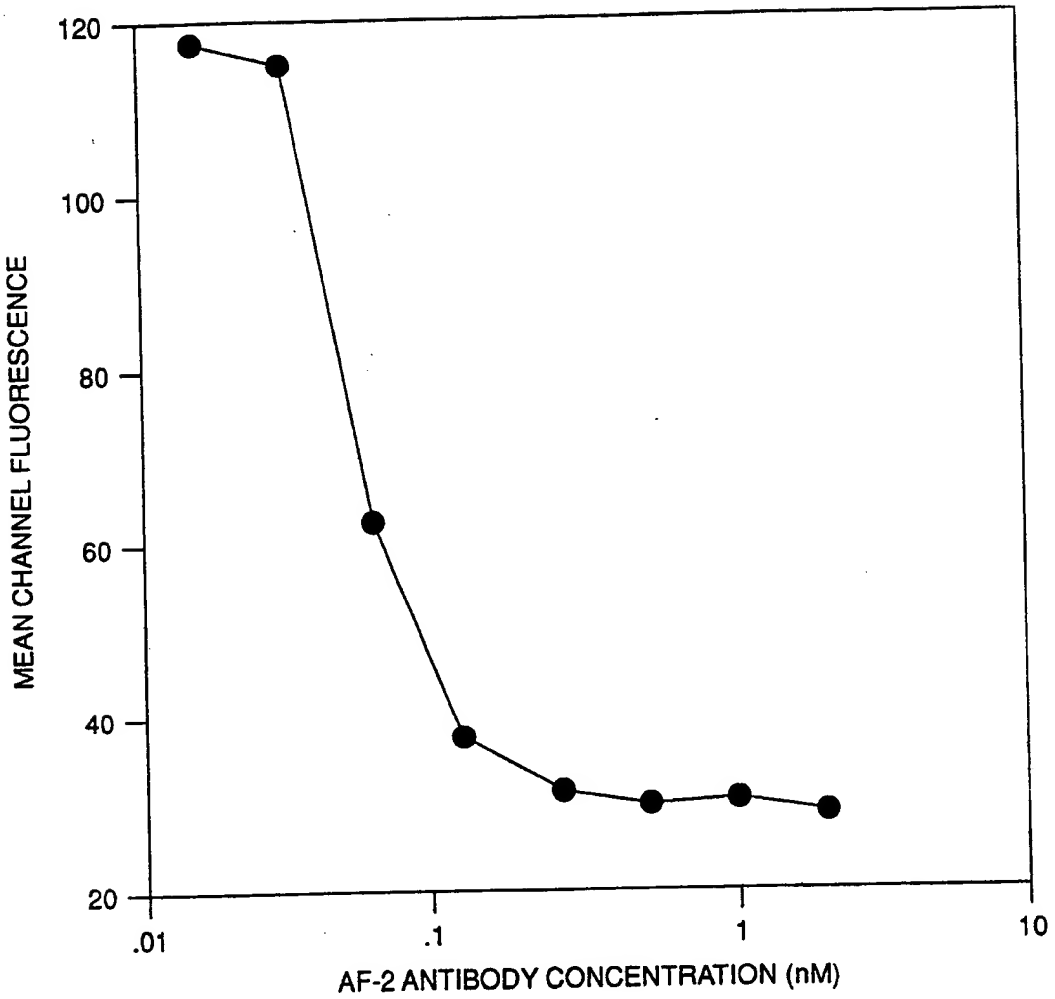
120
100
80
60
40
20
MEAN CHANNEL FLUORESCENCE
.01
.1
1
10
AF-2 ANTIBODY CONCENTRATION (nM)

FIG. 34

INTERNATIONAL SEARCH REPORT

International Application No. PCT/US91/09711

I. CLASSIFICATION OF SUBJECT MATTER (if several classification symbols apply, indicate all)[3]

According to International Patent Classification (IPC) or to both National Classification and IPC

IPC (5): A61K 35/14, 39/00; C07K 15/00
US CL : 530/387,391; 424/85.91

II. FIELDS SEARCHED

Minimum Documentation Searched[4]

Classification System	Classification Symbols
U.S.	530/387,391; 424/85.91

Documentation Searched other than Minimum Documentation to the extent that such Documents are included in the Fields Searched[6]

III. DOCUMENTS CONSIDERED TO BE RELEVANT[14]

Category*	Citation of Document,[16] with indication, where appropriate, of the relevant passages[17]	Relevant to Claim No.[18]
Y	Journal of Biological Chemistry, Volume 260, No. 22, issued 05 October 1985, Lambert et al, "Purified Immunotoxins That are Reactive with Human Lymphoid Cells", pages 12035-12041, see entire document.	10,33,59,60
Y	US, A, 4,845,198 (Urdal et al) 04 July 1989, see columns 8-12.	1-9
Y	Journal of Experimental Medicine, Volume 162, issued July 1985, Kirkman et al, "Administration of an Anti-Interleukin 2 Receptor Monoclonal Antibody Prolongs Cardiac Allograft Survival in Mice", pages 358-362, see entire document.	1-9
Y	The Journal of Immunology, Volume 126, No.4, issued 04 April 1981, Uchiyama et al, "A Monoclonal Antibody (Anti-Tac) Reactive with Activated and Functionally Mature Human T Cells", pages 1393-1397, see pages 1393-1394...	1-10
Y	Nature, Volume 321, issued 29 May 1986, Jones et al, "Replacing the Complementarity Determining Regions in a Human Antibody with Those from a Mouse", pages 522-525, see pages 523-525.	1-9, 11-32, 34-58

* Special categories of cited documents:[15]

"A" document defining the general state of the art which is not considered to be of particular relevance
"E" earlier document but published on or after the international filing date
"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)
"O" document referring to an oral disclosure, use, exhibition or other means
"P" document published prior to the international filing date but later than the priority date claimed
"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step
"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"&" document member of the same patent family

IV. CERTIFICATION

Date of the Actual Completion of the International Search[2]	Date of Mailing of this International Search Report[2]
06 FEBRUARY 1992	27 FEB 1992
International Searching Authority[1]	Signature of Authorized Officer[20]
ISA/US	Lila Feisee

Form PCT/ISA/210 (second sheet)(May 1986) ß

FURTHER INFORMATION CONTINUED FROM THE SECOND SHEET

Y	Procedings of the National Academy of Sciences, Volume 86, issued December 1989, Queen et al, "A Humanized Antibody that Binds to the Interleukin-2 Receptor", pages 10029-10033, see entire document.	1-60
Y	WO, A, 88-07869 (Billiau et al) 20 October 1988. See the entire abstract.	50-58
Y	Journal of Interferon Research, Volume 10, No. 2, issued 1990, Jarpe et al, "Structure of an Epitope in an Immunodominant Region of the Interferon-gamma, Molecule that is Involved in Receptor Interaction", pages 243-52, see the entire abstract.	50-58

V. ☐ OBSERVATIONS WHERE CERTAIN CLAIMS WERE FOUND UNSEARCHABLE[1]

This international search report has not been established in respect of certain claims under Article 17(2) (a) for the following reasons:

1. ☐ Claim numbers _, because they relate to subject matter (1) not required to be searched by this Authority, namely:

2. ☐ Claim numbers _, because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out (1), specifically:

3. ☐ Claim numbers _, because they are dependent claims not drafted in accordance with the second and third sentences of PCT Rule 6.4(a).

VI. ☐ OBSERVATIONS WHERE UNITY OF INVENTION IS LACKING[2]

This International Searching Authority found multiple inventions in this international application as follows:

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims of the international application.

2. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims of the international application for which fees were paid, specifically claims:

3. ☐ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claim numbers:

4. ☐ As all searchable claims could be searched without effort justifying an additional fee, the International Search Authority did not invite payment of any additional fee.

Remark on protest

☐ The additional search fees were accompanied by applicant's protest.

☐ No protest accompanied the payment of additional search fees.

III. DOCUMENTS CONSIDERED TO BE RELEVANT (CONTINUED FROM THE SECOND SHEET)

Category*	Citation of Document,[16] with indication, where appropriate, of the relevant passages [17]	Relevant to Claim No.[18]
Y	Journal of Virology, Volume 62, No. 9, issued 1988, Britt et al, "Induction of Complement -dependant and independent Neutralizing Antibodies by Recombinant-derived Human Cytomegalovirus gp55-116(gB)", pages 3309-3318, see the entire abstract.	34-49
Y	Dist. Markers, Volume 5, No. 4, issued 1987, Favaloro et al, "Characterization of Monoclonal Antibodies to the Human Myeloid-differentiation Antigen, 'gp67' (CD33)", pages 215-225, see the entire abstract.	23-32
Y	Antiviral Research, Volume 10, No. 6, issued 1988, Bernstein et al, "Antibody to Cloned HSV Glycoproteins B and D Plus Adult Human leukocytes Protect Neonatal Mice from Lethal HSV Infection", pages 279-287. See the entire abstract.	11-22